Information Disclosure Statement

Regarding the requirement for an information disclosure statement (IDS), Applicants respectfully point out that an information disclosure statement including form PTO-1449 listing 6 references was filed with the PTO on 16 August 2000 and was received by the PTO on 21 August 2000. Copies of the information disclosure statement and postcard are attached. If the Examiner has not received the listed references, Applicants will forward additional copies of the references upon request.

Claim Rejections

Rejections Under 35 USC § 101

Claim 1 stands rejected under 35 USC § 101 as being drawn to subject matter with no apparent or disclosed specific and substantial credible utility. Applicants respectfully traverse.

The Office Action expresses that the specification fails to demonstrate involvement of R0101 (p15PAF) protein in a particular physiological process that an artisan would wish to manipulate. The Action further expresses that there is an absence of knowledge as to the natural ligands of R0101 and as a result there is no patentable use for it.

Review of the Specification: R0101 Ligands Include Well-Known Cell Cycle and DNA Synthesis Proteins

The specification clearly establishes that R0101 possesses a conserved PCNA-binding motif, as outlined in Figure 2. Further, the specification clearly establishes that R0101 is localized to the nucleus where PCNA and p21 are found, as shown in Figure 4. In addition, the specification establishes that R0101 can bind to the nuclear proteins PCNA, cdk2, and cdk3 in a yeast two hybrid assay. Moreover, the specification establishes that a variant of R0101 with altered amino

acid sequence through the conserved PCNA-binding motif cannot bind to PCNA. Finally, the specification establishes that R0101 can disrupt the binding of p21 to PCNA, and that p21 can disrupt the binding of R0101 to PCNA, as shown in Figure 6. Thus, contrary to the suggestion of the Office Action, the present specification describes a ligand of R0101, particularly PCNA, and further describes competitive binding to the PCNA ligand with p21.

Nature of R0101 Binding Partner "PCNA", and Competitive Binding Moiety "p21" Supports Well-Established Utility for R0101

Regarding the R0101 ligand PCNA, and the competitive binding protein p21, Applicants respectfully point out that much is known about these molecules and that compositions and methods for modulating their interaction and activity have immediately obvious "real world" utility.

PCNA plays an essential role in DNA synthesis, at least in part due to its function as a processivity factor for DNA polymerases delta and epsilon (see Kelman, Oncogene 14:629-640, 1997, a copy of which is enclosed as Exhibit A). p21 is an inhibitor of cell cycle progression which coordinately regulates DNA synthesis and kinase activity underlying cell cycle progression. The interaction of p21 (through the C-terminus) with PCNA inhibits DNA synthesis, while the interaction of p21 (through the N-terminus) with cdk-cyclins inhibits their kinase activity and advancement of the cell cycle (Kelman, supra).

The p21 gene is directly regulated by the wild-type p53 proto-oncogene and is able to suppress tumor cell growth in culture (El-Deiry et al., Cell 75:817-825, 1993, a copy of which is attached as Exhibit B). This suggests that p21 is one of the major downstream effectors of p53 in the pathway of growth suppression and cell cycle regulation which appears to be dysregulated in a large number of cancers. In addition, forced expression of PCNA anti-sense RNA in proliferating cells suppresses DNA replication and cell cycle progression (Jaskulski et al., Science, 240:1544-1546, 1988, a copy enclosed as Exhibit C).

The molecular and functional interactions of p21 and PCNA, and the ability of these factors to regulate cell cycle progression and DNA synthesis provides ample support that compositions and methods for modulating a p21-PCNA interaction are desirable. Accordingly, the R0101 cell cycle protein has a well-established utility.

Applicants Evidentiary Burden to Support Asserted Utility is Met

Without admission of a prima facie showing for lack of utility, Applicants consider, arguendo, the requirements for responding to such a prima facie showing.

Regarding Applicants evidentiary burden in response to a prima facie showing of lack of utility, the MPEP states at §2107.01, VII:

> Furthermore, the applicant does not have to provide evidence sufficient to establish that an asserted utility is true "beyond a reasonable doubt". In re Irons, 340 F.2d 974, 978, 144 USPQ 351, 354 (CCPA 1965). Instead, evidence will be sufficient if, considered as a whole, it leads a person of ordinary skill in the art to conclude that the asserted utility is more likely than not true.

The specification adequately supports that R0101 binds to PCNA and may be used to modulate PCNA binding to p21. Further, the preceding discussion adequately supports that the p21 and PCNA interaction underlies a physiological process, the manipulation of which has "real world" utility.

The Office Action does not present evidence that the asserted utility of R0101 is an incredible utility, that R0101 can not bind PCNA, or that R0101 can not modulate the binding of PCNA to p21. Further, the Office Action does not provide evidence that the interaction of PCNA and p21 is not important in cell cycle control, and that modulation of this interaction is undesirable.

Accordingly, Applicants submit that the asserted utility is credible, and respectfully request withdrawal of the rejection.

Rejections Under 35 U.S.C. § 112, first paragraph - how to use

Claim 1 stands rejected under 35 U.S.C. § 112, first paragraph. The Office Action expresses that since a patentable utility for the invention is not set forth, the specification fails to teach one of reasonable skill in the art how to make and use the invention for an asserted patentable utility. Applicants respectfully traverse.

Applicants submit that the instant invention has well-established and fully disclosed utility, for reasons set forth above. Applicants request consideration of the remarks offered above as they pertain to the rejection under 35 U.S.C. § 112, first paragraph, and respectfully request withdrawal of the rejection.

Rejections Under 35 U.S.C. § 112, first paragraph - enablement

Claim 1stands further rejected under 35 U.S.C. § 112, first paragraph, as lacking enablement. Particularly, the Office Action expresses that the a method of screening for a candidate agent which binds a cell cycle protein is not enabled. Applicants respectfully traverse.

Applicants point out that claim 1 has been cancelled, and that the new claims are not drawn to such a screening method. Particularly, Claim 10 is drawn to a method for screening for a candidate agent capable of binding to a protein comprising an amino acid sequence having at least about 95% identity to the amino acid sequence set forth in SEQ ID NO:2. Further, Claims 11-14 are drawn to methods for screening involving the use of a protein comprising an amino acid sequence having at least about 95% identity to the amino acid sequence set forth in SEQ ID NO:2.

Applicants submit that the newly submitted claims 10-14 are fully enabled by the instant specification. Applicants respectfully request withdrawal of the rejection and allowance of the newly pending claims.

Respectfully submitted,

FLEHR HOHBACH TEST
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Dated: 1 June 2001

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VERSION WITH MARKINGS TO SHOW CHANGES MADE

~~1. A method for screening for a bioactive agent capable of binding to a cell cycle protein R0101, said method comprising:~~

~~a) combining a cell cycle protein R0101 and a candidate bioactive agent; and~~

~~b) determining the binding of said candidate bioactive agent to said cell cycle protein R0101.~~

~~2. A method for screening for a bioactive agent capable of interfering with the binding of a cell cycle protein R0101 and a PCNA protein, said method comprising:~~

~~a) combining a cell cycle protein R0101, a candidate bioactive agent and a PCNA protein; and~~

~~b) determining the binding of said cell cycle protein R0101 and said PCNA protein.~~

~~3. A method according to Claim 2, wherein said cell cycle protein R0101 and said PCNA protein are combined first.~~

~~4. A method for screening for a bioactive agent capable of modulating the activity of cell cycle protein R0101, said method comprising:~~

~~a) adding a candidate bioactive agent to a cell comprising a recombinant nucleic acid encoding a cell cycle protein R0101; and~~

~~b) determining the effect of said candidate bioactive agent on said cell.~~

~~5. A method according to Claim 4, wherein a library of candidate bioactive agents is added to a plurality of cells comprising a recombinant nucleic acid encoding a cell cycle protein R0101.~~

~~6. An antibody to a cell cycle protein R0101.~~

~~7. The antibody of Claim 6 wherein said antibody is a monoclonal antibody.~~

~~8. The antibody of Claim 6 wherein said antibody reduces or eliminates the biological function of said cell cycle protein R0101.~~

~~9. A method of diagnosing cancer in an individual, said method comprising determining the level of expression of R0101 in a sample taken from an individual and comparing said level to a control which has a level which indicates there is no cancer, wherein an increase in said sample compared to said control indicates a diagnoses of cancer.~~

10. A method for screening for a bioactive agent capable of binding a protein, comprising:

a) combining said protein and a candidate bioactive agent; and

b) determining the binding of said candidate agent to said protein;

wherein said protein comprises an amino acid sequence having at least about 95% identity to the amino acid sequence set forth in SEQ ID NO:2.

11. A method of screening for a bioactive agent capable of modulating the binding of PCNA and p21, comprising:

a) combining PCNA, p21, a candidate bioactive agent, and a protein;

b) determining the binding of p21 to PCNA in the presence and absence of candidate agent;

wherein said protein comprises an amino acid sequence having at least about 95% identity to the amino acid sequence set forth in SEQ ID NO:2.

12. A method of screening for a bioactive agent capable of modulating the binding of PCNA and p21, comprising:

a) combining PCNA, p21, a candidate bioactive agent, and a protein;

b) determining the binding of PCNA to said protein in the presence and absence of candidate agent;

wherein said protein comprises an amino acid sequence having at least about 95% identity to the amino acid sequence set forth in SEQ ID NO:2.

13. A method of screening for a bioactive agent capable of modulating DNA synthesis, comprising:

a) combining PCNA, p21, a candidate bioactive agent, and a protein; and

b) determining the binding of p21 to PCNA in the presence and absence of candidate agent;

wherein said protein comprises an amino acid sequence having at least about 95% identity to the amino acid sequence set forth in SEQ ID NO:2.

14. A method of screening for a bioactive agent capable of modulating DNA synthesis, comprising:

a) combining PCNA, p21, a candidate bioactive agent, and a protein; and

b) determining the binding of PCNA to said protein in the presence and absence of candidate agent;

wherein said protein comprises an amino acid sequence having at least about 95% identity to the amino acid sequence set forth in SEQ ID NO:2.

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REVIEW

PCNA: structure, functions and interactions

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Proliferating cell nuclear antigen (PCNA) plays an essential role in nucleic acid metabolism as a component of the replication and repair machinery. This toroidal-shaped protein encircles DNA and can slide bi-directionally along the duplex. One of the well-established functions for PCNA is its role as the processivity factor for DNA polymerase δ and ε. PCNA tethers the polymerase catalytic unit to the DNA template for rapid and processive DNA synthesis. In the last several years it has become apparent that PCNA interacts with proteins involved in cell-cycle progression which are not a part of the DNA polymerase apparatus. Some of these interactions have a direct effect on DNA synthesis while the roles of several other interactions are not fully understood. This review summarizes the structural features of PCNA and describes the diverse functions played by the protein in DNA replication and repair as well as its possible role in chromatin assembly and gene transcription. The PCNA interactions with different cellular proteins and the importance of these interactions are also discussed.

Keywords: DNA replication; DNA repair; cell cycle; p53; PCNA; protein-DNA interaction

Introduction

Proliferating cell nuclear antigen (PCNA) was identified independently by two different groups. Miyachi *et al.* (1978) initially identified an auto-antigen in patients with systemic lupus erythematosis, which they named PCNA because the protein was observed in the nucleus of dividing cells. At about the same time Bravo and Celis (1980) used two dimensional gel electrophoresis to identify a protein which was synthesized during the S-phase of the cell cycle which they named cyclin. Subsequent work demonstrated that PCNA and cyclin are the same 29 kDa protein (Mathews *et al.*, 1984). This protein is now called PCNA; the term cyclin is now used to describe a family of proteins involved in cell cycle regulation. Genes which encode the PCNA protein have been isolated from several eukaryotes and archebacteria and the predicted amino acid sequences have been found to be highly conserved (Bult *et al.*, 1996; Kelman and O'Donnell, 1995a). The chromosomal locations of PCNA genes from several species have been determined (e.g. Henderson *et al.*, 1994; Ku *et al.*, 1989), as have several PCNA pseudogenes (Ku *et al.*, 1989; Yamaguchi *et al.*, 1991).

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Extensive studies have been conducted on PCNA and its functions in the past several years, and PCNA has been found to play a critical role in several biological processes that appear disparate but have in common a role in DNA metabolism. PCNA is an essential component of the DNA replication machinery, functioning as the accessory protein for DNA polymerase δ (Polδ), required for processive chromosomal DNA synthesis, and DNA polymerase ε (Polε). PCNA is also required for DNA recombination and repair. In addition, PCNA was shown to interact with cellular proteins involved in cell cycle regulation and check point control. Furthermore, the three-dimensional structures of yeast and human PCNA have been determined and several structure-function relationships have been established.

The aim of this article is to summarize our current understanding of the diverse functions played by PCNA in DNA metabolism. The association between PCNA and different cellular proteins will be described, as well as the roles played by these interactions.

Structure

The biochemical data

PCNA is the processivity factor of Polδ and thus is the functional homologue of other processivity factors, the β subunit of the *Escherichia coli* DNA polymerase III holoenzyme (Pol III) and the product of gene-45 of bacteriophage-T4. Similarities in the function of these proteins gave the first indication of the structure of PCNA.

PCNA has been described as a 'sliding clamp'. The first evidence for the sliding clamp structure came from the study of the β subunit of *E. coli* Pol III. In a series of elegant experiments it was shown that the β subunit bound tightly to nicked circular plasmid but readily dissociated upon linearization of the plasmid via sliding over the ends (Stukenberg *et al.*, 1991; Yao *et al.*, 1996; reviewed in Kelman and O'Donnell, 1995b). These results suggested that the β subunit binds DNA in a topological fashion, by encircling it. The g45 protein has a similar structure. Electron microscopy studies showed that g45 protein forms structures that resemble 'hash-marks' on DNA, and it appears that the DNA is threaded through the protein (Gogol *et al.*, 1992).

Several other lines of biochemical data also supported the idea that PCNA forms a ring around DNA. In the absence of the clamp loader (RF-C) (the complex that assembles the ring around duplex DNA; discussed below) it was demonstrated that PCNA can support processive replication by Polδ on linear DNA with a

double stranded end, but not on a closed circular template (Burgers and Yoder, 1993). These results suggested that PCNA could thread itself onto the end of the dsDNA molecule and slide along the duplex until it reached the 3′ end, where it interacted with Polδ to initiate processive DNA synthesis. Crosslinking experiments were used in a different strategy to demonstrate the sliding property of PCNA (Tinker *et al.*, 1994). After assembly around circular plasmid DNA, PCNA could be crosslinked to the DNA. Upon linearization of the plasmid, however, PCNA could no longer be crosslinked to the template, suggesting it has sliding properties (Tinker *et al.*, 1994). Experiments similar to these used to demonstrate the sliding of the *E. coli* β subunit were performed with human PCNA. This study showed that PCNA could bind nicked circular plasmid but slid off the ends upon linearization (Yao *et al.*, 1996).

The three dimensional structure

The crystal structure analysis of the *E. coli* β dimer showed it to be a ring shaped protein with an inner diameter sufficient to accommodate a dsDNA molecule (Kong *et al.*, 1992). In the paper describing the three dimensional structure of β the authors predicted that PCNA would have a similar structure (Kong *et al.*, 1992). In fact, the PCNA monomer is only 2/3 the size of the β subunit but was shown to form a trimer in solution (Bauer and Burgers, 1988a; Brand *et al.*, 1994; Yao *et al.*, 1996). It was therefore suggested that PCNA would form a trimeric ring (Kong *et al.*, 1992). Indeed, the overall structures of the human (Gulbis *et al.*, 1996) and yeast (Krishna *et al.*, 1994) PCNAs are very similar to each other and to the *E. coli* β subunit. The rings are superimposeable; each ring has similar dimensions and each has a diameter of the central cavity large enough to accommodate a dsDNA molecule (Krishna *et al.*, 1994; reviewed in Kuriyan and O'Donnell, 1993; Kelman and O'Donnell, 1995a).

The PCNA trimer forms a ring with an inner diameter of 34Å; this is sufficient to accommodate B-form DNA (Figure 1). The trimeric ring has sixfold symmetry as each monomer contains two globular domains. Each domain contains two α helices which line the central cavity and are perpendicular to the DNA. The α helices are supported by a continuous layer of nine β sheet structures all around the outside. Surprisingly, although the domains in each monomer do not have sequence similarity they are nearly identical in three-dimensions (Krishna *et al.*, 1994). It is interesting to note that the dimer of the *E. coli* β subunit is also composed of six domains while each monomer contains three globular domains (Kong *et al.*, 1992), and that several other enzymes involved in DNA metabolism share a similar six domain structure (Kelman *et al.*, 1995).

In all species studied, PCNA is an acidic protein with low pI (Kelman and O'Donnell, 1995a). The charge distribution on the ring is asymmetric. There is a strong negative electrostatic potential in the outer surface and a net positive electrostatic potential in the central cavity (Krishna *et al.*, 1994). The negatively charged surface may prevent nonspecific interaction with DNA. Upon assembly around DNA by the clamp loader (see below) the positively charged central cavity may stabilize the interactions between PCNA and DNA.

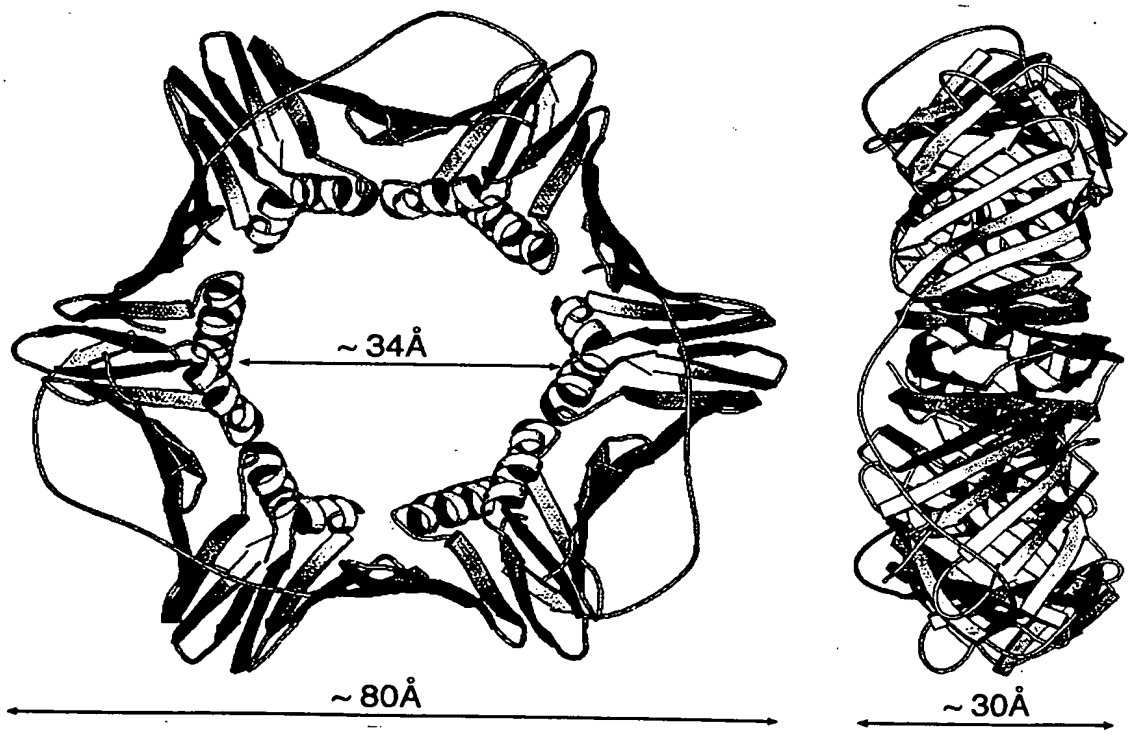

Figure 1 Structure of human PCNA. Left panel: Front view. Twelve α helices line the inner cavity and the outside perimeter is a continuous layer of β sheet structure. The inner and outer diameters of the ring are shown. Right panel: Side view. The width of the ring is shown

The PCNA trimer is stable both in solution and on DNA. In solution, the K_d of PCNA dissociation into monomers is 20 nM (Podust *et al.*, 1995; Yao *et al.*, 1996), and the trimer remains stable at 500 mM NaCl. PCNA is also stable on DNA with an observed half life of 22 min (Podust *et al.*, 1995; Yao *et al.*, 1996). Several forces may be involved in the stability of the ring. The X-ray structure revealed that the interfaces between the promoters are formed by parallel β sheets composed of nine residues from each monomer (Krishna *et al.*, 1994). The stabilization of the ring results from hydrogen bonding between the β sheets, and may additionally include the formation of a hydrophobic core and an ion pair. In yeast PCNA there are four pairs of hydrophobic residues which form a hydrophobic core and one putative ion pair (Krishna *et al.*, 1994; Kelman and O'Donnell, 1995a).

Apparently, not all of the above putative interactions are equally important for the stabilization of the ring. It was demonstrated that a single amino acid substitution at the dimer interface where Tyr was replaced by Ala causes PCNA to become a monomer (Jonsson *et al.*, 1995). It was also shown that this monomeric PCNA cannot be assembled around DNA and fails to support replication by Polδ (Jonsson *et al.*, 1995).

The integrity of the β sheets may also be important for the stability of the ring. A single amino acid substitution in the β sheet lining the interface where Ser is replaced by Pro resulted in the monomerization of the protein (Ayyagari *et al.*, 1995). Pro residues are rarely found in β sheet structures (Smith *et al.*, 1994a) and would likely effect the integrity of the sheet structure. Furthermore, in the *E. coli* β subunit a similar substitution (Leu replaced by Pro) in the β sheet structure prevented the formation of a β dimer (Yao *et al.*, 1996). An Ala substitution at the same position does not affect the integrity of the β ring, which remains stable as a dimer (Z Kelman and M O'Donnell, unpublished observation). Although the three-dimensional structure of PCNA has been solved and several amino acid residues have been identified as being important for interface stability, further research is needed to shed light on the function of specific amino acid residues and the exact nature of the interaction that stabilize the protein structure.

Alternative structure of PCNA

Both mammalian and yeast PCNAs have been shown to form trimers in solution (Bauer and Burgers, 1988a; Yao *et al.*, 1996) and were crystallized as trimers (Krishna *et al.*, 1994; Gulbis *et al.*, 1996). PCNA, however, may also exist as a dimeric ring. During somatic embryogenesis of *Daucus carota*, the carrot, two distinct PCNA genes are expressed (Hata *et al.*, 1992). One encodes a typical size PCNA (264 amino acids) while the other encodes a longer form (365 amino acids). The protein encoded by the long form of PCNA has a molecular mass of 40.1 kDa. This size is similar to the size of the prokaryotic β subunit and thus might form a dimeric ring. Furthermore, in early *Xenopus laevis* oogenesis a large PCNA transcript can be detected and a 43 kDa protein was shown to cross-react with PCNA antibody (Leibovici *et al.*, 1990). It was suggested that these putative long forms of PCNA are important for rapid chromosomal replication needed in early embryogenesis (Kelman and O'Donnell, 1995c) and/or for DNA repair (Stillman, 1996).

As described above, PCNA exhibits sixfold symmetry as a result of having two globular domains in each monomer (Figure 1). Several other proteins which are involved in DNA metabolism (e.g. helicase, RuvB) also have sixfold symmetry and form a hexameric ring (Kelman *et al.*, 1995). It is interesting to note that a 19 kDa protein, which cross reacts with anti-PCNA antibodies, has been identified in the phytoplankton *Skeletonema costatum* (Lin *et al.*, 1994). If this protein is indeed a PCNA homologue, and not a degradation product, then it might form a hexameric ring. Thus PCNA may exist in several forms having sixfold symmetry; as a hexamer, trimer, or dimer.

Function

PCNA plays important roles in nucleic acid metabolism. The protein is essential for DNA replication, is involved in DNA excision repair, has been suggested to be involved in chromatin assembly, and in several instances has been shown to be involved in RNA transcription.

DNA replication

The best understood function of PCNA, to date, is its role in DNA replication. PCNA plays an essential role in DNA replication as the auxillary protein (processitivity factor) of Polδ, the enzyme responsible for the replication of the chromosomal DNA (Figure 2). PCNA is also the processivity factor of Polε. The role of this enzyme *in vivo* is not yet clear (discussed below).

PCNA was isolated as a protein with elevated levels during S-phase (Bravo and Celis, 1980). In quiescent and senescent cells there are very low levels of PCNA mRNA and protein. Upon serum or mitogen stimulation of cells there is a several fold increase in the level of PCNA transcript and protein shortly before DNA synthesis (Celis *et al.*, 1988 and references therein). In contrast to quiescent cells, in actively growing cells PCNA is constitutively expressed throughout the cell cycle. Studies have shown, however, that the protein is localized to the nucleus only in cells that are in the S-phase of the cell cycle (Takasaki *et al.*, 1981; Bravo and Macdonald-Bravo, 1985, 1987) or in u.v.-irradiated non-S-phase cells (DNA repair) (Celis and Madson, 1986; Toschi and Bravo, 1988).

These early studies of PCNA expression and cellular localization during DNA synthesis suggested that the protein either plays a role in DNA replication directly, as a part of the replication machinery, or indirectly as a regulatory factor. Soon thereafter the direct role of PCNA in DNA replication was elucidated.

PCNA was identified as a component required for the *in vitro* replication of SV-40 (Prelich *et al.*, 1987a; reviewed in Challberg and Kelly, 1989). In an *in vitro* replication assay PCNA could be replaced by a previously described protein (Prelich *et al.*, 1987b). This protein was originally identified as an auxillary protein of Polδ, by its ability to enable purified Polδ to replicate a template with long single stranded regions

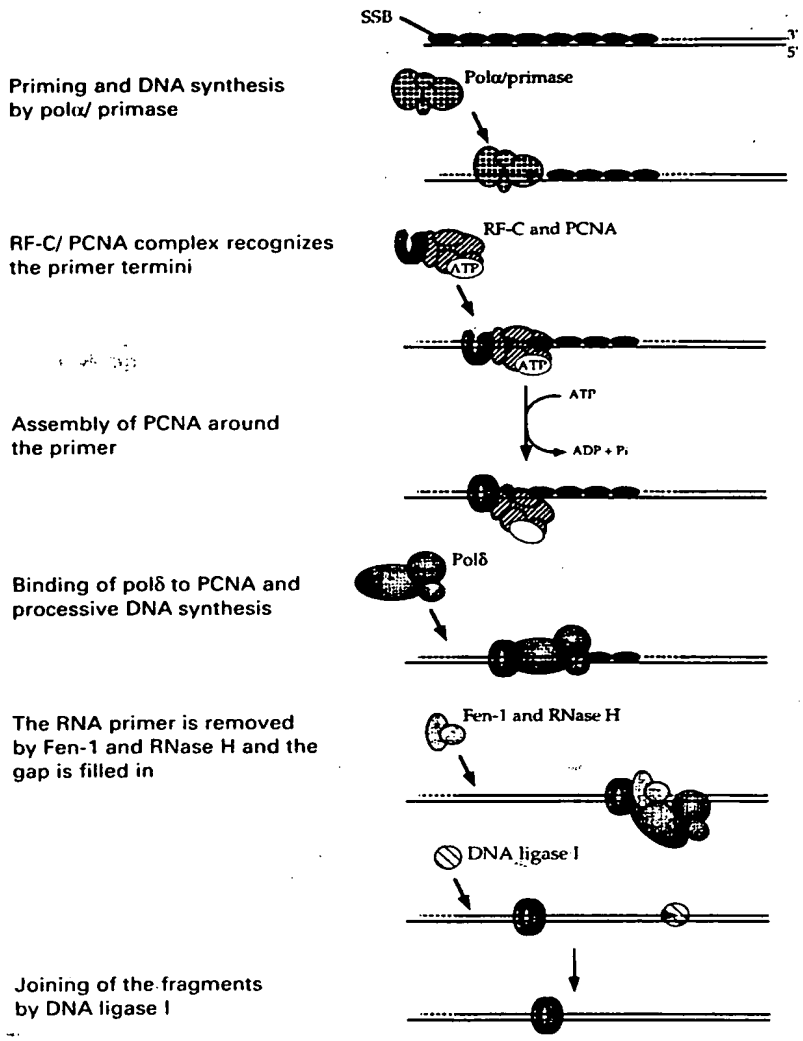

Figure 2 Synthesis of an Okazaki fragment during the replication of the lagging strand. The leading strand is replicated continuously by PCNA and Polδ and for simplicity was omitted from the Figure. Several of the steps have been deduced from studies of *E. coli* and phage-T4 replicases

(Tan *et al.*, 1986). Furthermore, using Western blotting it was shown that antibodies generated against PCNA recognized the auxillary protein of Polδ and that the two proteins share similar N-terminal amino acid sequence (Bravo *et al.*, 1987; Prelich *et al.*, 1987b). The biochemical studies together with the immunological data and the amino acid sequence analysis confirmed that the auxillary protein of Polδ and PCNA were identical and established the role of PCNA in DNA replication.

These initial observations were soon followed by other *in vitro* and *in vivo* studies that elucidated the function of PCNA. One of the early functions demonstrated for PCNA is its important role in coordinating leading and lagging strand synthesis (Prelich and Stillman, 1988). Using fractionated cell extract, in an SV-40 *in vitro* replication assay, it was shown that in the absence of PCNA leading strand DNA synthesis was abolished and lagging strand DNA replication was abnormal (Prelich and Stillman, 1988). Cell extracts and purified proteins were also used to further establish the role of PCNA as the processivity factor of Polδ. Polδ has very poor processivity by itself. Its processivity, however, is dramatically increased in the presence of RF-C and PCNA (Downey *et al.*, 1990 and references therein). Furthermore, antibodies generated against PCNA inhibited Polδ dependent *in vitro* replication but not replication by other polymerases (Tan *et al.*, 1987).

The role of PCNA *in vivo* was the focus of several studies. Expression of PCNA anti-sense RNA in exponentially growing cells caused a suppression of DNA replication and cell cycle progression (Jaskulski *et al.*, 1988). Yeast cells were also used to study the function of PCNA. Deletion of the PCNA gene in both the budding yeast, *Saccharomyces cerevisae* (Bauer and Burgers, 1990) and in fission yeast, *Schizosaccharomyces pombe* (Waseem *et al.*, 1992), demonstrated that PCNA is an essential protein and is required for DNA replication.

Much of our understanding of the molecular mechanism by which PCNA functions as the processivity factor for Polδ comes from the elaborate studies on the *E. coli* counterpart of PCNA, the β subunit of Pol

III. Similar to PCNA, β is a ring shaped protein which encircles DNA. The ring is assembled around the DNA by a clamp loader complex that recognizes the primer terminus and loads the ring onto the double stranded region of it. This clamp loader is the γ-complex subassembly of Pol III and it shares functional and probably structural similarities with the RF-C complex of Polδ holoenzyme (O'Donnell *et al.*, 1993; Cullmann *et al.*, 1995; reviewed in Kelman and O'Donnell, 1994; Stillman, 1994). After loading β onto DNA the polymerase catalytic unit replaces the γ-complex in the binding to the β ring (Naktinis *et al.*, 1996, reviewed in Herendeen and Kelly, 1996). The binding of the polymerase to the ring tethers it to the template for processive DNA synthesis. It was recently shown that the human RF-C protein operates in a similar manner and that following the loading of PCNA onto the DNA template Polδ can bind to it to initiate processive replication (Figure 2) (Yao *et al.*, 1996).

Recently, an additional role for PCNA in DNA replication has emerged. FEN-1, a protein which is involved in the maturation of an Okazaki fragment, was shown to interact with PCNA (discussed below). The binding to PCNA stimulates the 5′ to 3′ exonuclease activity of FEN-1, a function which is important for the formation of a continuous lagging strand. Therefore, PCNA plays a role in DNA synthesis not only as part of the polymerase holoenzyme but also in the final steps leading to the formation of a complete DNA duplex (Figure 2).

DNA repair

Early immunohistochemical studies using PCNA antibodies to study the distribution of the protein within the cell suggested a role for PCNA in DNA repair. The nucleus stained strongly with anti-PCNA antibodies following DNA damage by u.v.-irradiation although the cells were not in S-phase. This observation suggested the involvement of the protein in DNA repair processes (Celis and Madsen, 1986; Toschi and Bravo, 1988). Indeed, *in vitro* repair assays have demonstrated the involvement of PCNA in DNA excision and mismatch repair processes (Nichols and Sancar, 1992; Shivji *et al.*, 1992; Johnson *et al.*, 1996; Umar *et al.*, 1996). Furthermore, studies conducted on the *Drosophila melanogaster mus209* mutant demonstrated an *in vivo* role for PCNA in DNA repair (Henderson *et al.*, 1994).

Thus, PCNA is involved in both DNA replication and repair. The mechanism by which PCNA is involved in repair remains to be elucidated, however. Some evidence suggests that PCNA plays different roles in replication and repair. A study of different mutant forms of PCNA revealed that some affect DNA repair but not replication (Ayyagari *et al.*, 1995). Several studies have indicated that Polε plays a major role in DNA repair (Nishida *et al.*, 1988; Wang *et al.*, 1993; Aboussekhra *et al.*, 1995; reviewed in Wood, 1996). PCNA interacts with both Polδ and Polε (described below). Therefore, interaction between Polε and PCNA may be important for DNA repair while interaction with Polδ may be important only during chromosomal DNA replication.

In the last few years a new link between PCNA and the cellular response to DNA damage has been identified – the p53 protein. p53 is a transcription factor which is a key component of the cellular response to DNA damage, and has been called the 'guardian of the genome'. Exposing the cell to DNA damage causes an increase in the level of the active form of p53. High levels of p53 affect the transcription of several genes and bring about cell cycle arrest while cells that lack the protein fail to arrest after DNA damage (reviewed in Cox and Lane, 1995; Sanchez and Elledge, 1995; Bates and Vousden, 1996).

PCNA transcription is stimulated upon exposure of the cell to u.v.-irradiation (Zeng *et al.*, 1994). It was recently demonstrated that this elevation in PCNA expression might be mediated by the p53 protein, which was shown to activate transcription from the promoter of the PCNA gene (Shivakumar *et al.*, 1995; Morris *et al.*, 1996). Also, *in vivo* studies, conducted in yeast, suggested a direct role of PCNA in the check point mechanism via its interactions (directly or indirectly) with other, well established check point genes (Tournier *et al.*, 1996). The emerging picture is even more complex. Following DNA damage, p53 activates the expression of several genes. Two of these genes, p21 and Gadd45, have been shown to bind PCNA (Figure 3) (described below). Although an immunohistochemistry study suggested that p21 and PCNA interact during repair processes (Li *et al.*, 1996) the precise roles of the interactions between PCNA, p21 and Gadd45 in DNA repair are not yet clear.

Chromatin assembly

Recently, evidence for a role for PCNA in chromatin remodeling has been found. Studies conducted on the first few cell divisions following fertilization in the starfish *Asterina pectinifera* have suggested a role for PCNA in the assembly of chromatin (Nomura, 1994).

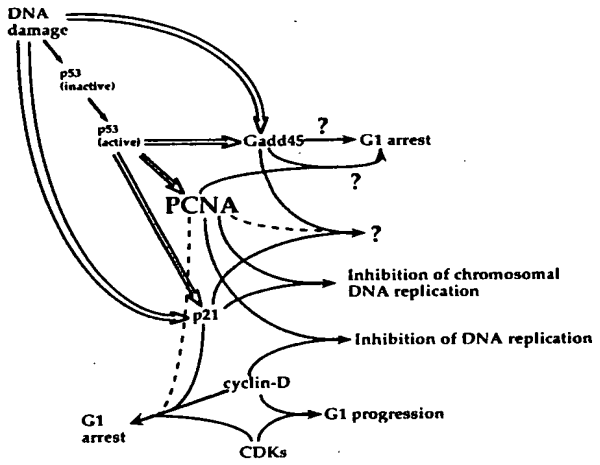

Figure 3 The role of PCNA and associated proteins upon DNA damage. Open arrows represent transcriptional regulation. Solid lines indicate protein-protein interactions. Question marks note unclear effects. Dashed lines are used to describe the interaction between PCNA and the cyclin-D-cdk complex because it has not been shown that PCNA is required in this complex to bring about G_1 arrest. It is also not yet clear if PCNA interacts with Gadd45 and p21 in a tertiary complex; it has been shown that PCNA can form complexes with each protein individually, and it has been suggested that p21 and Gadd45 compete for the binding to PCNA. See text for details

Using immunohistology with PCNA antibodies it was demonstrated that during S-phase PCNA is localized to specific regions on the DNA. These studies also suggested that during the first S-phase chromatin is relocated to the sites where PCNA is localized (Nomura, 1994). These observations imply a role for PCNA in the remodeling of chromatin structures during early embryogenesis. It is tempting to speculate that the long form of PCNA, identified in carrot embryos, (described above) might play a role in these processes.

A role for PCNA in chromatin assembly also came from studies conducted on a *D. melanogaster* mutant (Henderson *et al.*, 1994). This PCNA mutant, *mus209*, may have defects in chromatin remodeling. Using a marker gene located in a region of heterochromatin (transcriptionally inactive), it was demonstrated that the gene was transcriptionally active in the *mus209* background (Henderson *et al.*, 1994). This observation suggests that the mutation in PCNA affects the chromatin structure in the vicinity of the marker gene, making the region transcriptionally active (euchromatin).

These two *in vivo* studies indicate a role for PCNA role in the assembly of chromatin. It was previously suggested that a component of the replication fork might be involved in the chromatin assembly process (Smith and Stillman, 1989). PCNA may be that protein. The recent isolation of large numbers of PCNA mutants, mainly in yeast, will be a useful tool to further elucidate the role(s) of PCNA in this process.

RNA transcription

Does PCNA, a key replication protein, also play a role in gene transcription? Several well characterized examples exist for the involvement of viral encoded sliding clamps in RNA transcription. However, such a function has not yet been demonstrated for cellular PCNA.

The product of gene-45 of the *E. coli* bacteriophage-T4 is the functional homologue of PCNA. In a series of elegant experiments it was demonstrated that gene-45 protein plays an important role in the transcription of genes which are expressed in late stages of infection (Herendeen *et al.*, 1992). The gene-45 protein, in association with the gene-33 and gene-55 proteins, binds to the *E. coli* RNA polymerase and directs it to the promoters of the phage genes that are expressed in late stages of infection.

Another example of the role played by the sliding clamp in viral gene expression came from the study of the PCNA homologue from the *Autographa californica* nuclear polyhedrosis virus. Although the PCNA homologue encoded by this baculovirus is not essential for DNA replication, it has been shown to be important for the expression of several viral genes that are transcribed during late infection (Crawford and Miller, 1988; O'Reilly *et al.*, 1989).

These two examples imply that cellular encoded PCNA may have a similar function and would play a role in gene expression. Upon completion of DNA replication the PCNA ring may be left around the DNA (Yao *et al.*, 1996). These rings may then bind to RNA polymerase, directly or indirectly, and regulate gene expression. If such a mechanism exists in eukaryotic cells, likely genes for such regulation are genes encoding proteins which are essential for the G_2 or M-phases of the cell cycle. PCNA which is left on the DNA may serve as a 'marker' for the cell indicating the completion of DNA synthesis, and for the expression of specific genes.

Interactions with other proteins

In the last several years a number of proteins have been shown to associate with PCNA. Some of these proteins are directly involved in DNA replication and their interaction with PCNA has a direct role in DNA synthesis. Others, however, are proteins which are not a part of the replication apparatus but rather play diverse roles in cell cycle control and check point processes. Several of the known interactions between PCNA and these cellular components are summarized in Table 1.

Replication Factor C (RF-C)

RF-C, also called activator-1, is a complex composed of five subunits with molecular weights of 36, 37, 38, 40 and 128 kDa. RF-C functions as the clamp loader for PCNA. This complex is required for the efficient assembly of the PCNA trimer at the primer terminus in an ATP-dependent manner (Figure 2) (Lee and Hurwitz, 1990; Lee *et al.*, 1991; Tsurimoto and Stillman, 1990; Burgers, 1991; reviewed in Kelman and O'Donnell, 1994; Stillman, 1994). The RF-C complex recognizes the primer terminus and assembles the PCNA clamp around the primer. It was recently demonstrated that RF-C is also capable of removing PCNA from DNA upon the completion of an Okazaki fragment and thus may recycle PCNA for re-use at a new primer site (Yao *et al.*, 1996). The functions of RF-C as a clamp loader and unloader imply that it forms a physical contact with PCNA.

Table 1 Proteins known to interact with PCNA, and the roles of the interaction

PCNA interacts with	*Function of protein*	*Role of interaction with PCNA*
Replication Factor C	Loads and Unloads the sliding clamp	Loading/unloading of PCNA
DNA polymerase δ	The catalytic unit of DNA polymerase δ holoenzyme (DNA replication and repair)	Required for processive DNA synthesis
DNA polymerase ε	DNA polymerase (lagging strand polymerase [?], DNA repair, check point control)	Required for processive DNA synthesis
Fen-1	Flap endonuclease (Okazaki fragment maturation)	Stimulates the activity of Fen-1
Gadd45	Unknown; induced upon DNA damage	Unknown
D-type cyclins	G1 cyclins	Inhibits DNA replication
p21/Cip1 CDK inhibitor	Cyclin-dependent kinase inhibitor	Inhibits processive DNA synthesis
RNA polymerase	RNA polymerase	Required for viral late gene transcription (baculovirus)

Indeed, RF-C was shown to form a complex with PCNA at the primer terminus (Tsurimoto and Stillman, 1991), and complex formation stimulated the ATPase activity of RF-C (Tsurimoto and Stillman, 1990; Fukuda *et al.*, 1995). A direct interaction between the 40 kDa subunit of human RF-C and PCNA was demonstrated using purified proteins and an affinity column (Pan *et al.*, 1993). Genetic data from studies of *S. cerevisae*, however, suggest that there may be an interaction between PCNA and the large subunit of RF-C (McAlear *et al.*, 1994; Ayyagari *et al.*, 1995). Several mutations in the PCNA gene have been shown to suppress mutations in the 128 kDa subunit of RF-C (McAlear *et al.*, 1994; Ayyagari *et al.*, 1995). Indeed, studies conducted on truncated forms of the large subunit demonstrated a direct interaction between RF-C and PCNA (Fotedar *et al.*, 1996). It was also shown that two different regions of the 128 kDa subunit interact with PCNA and the DNA template (Fotedar *et al.*, 1996). Based on the recent observation that the RF-C complex can unload PCNA from DNA (Yao *et al.*, 1996) it may be that different subunits of RF-C interact with PCNA during the loading and unloading reactions.

DNA polymerase δ

PCNA is the processivity factor of Polδ (Bravo *et al.*, 1987; Prelich *et al.*, 1987b; Tan *et al.*, 1986; Downey *et al.*, 1990). *In vitro* studies demonstrated a significant stimulation of Polδ processivity (Waga and Stillman, 1994) by PCNA, suggesting an interaction between PCNA and Polδ.

Polδ contains at least two subunits with apparent molecular weights of 125 and 50 kDa (Wang, 1996 and references therein). Polδ may exist as a trimer or even tetramer (Jiang *et al.*, 1995a) but the additional subunits have not yet been purified and characterized. The 125 kDa subunit of yeast Polδ was shown to interact with PCNA when analysed using a sizing column (Bauer and Burgers, 1988b) and human Polδ was shown to bind to PCNA when co-expressed in baculovirus-infected cells (Zhang *et al.*, 1995). Using a truncated form of Polδ it was demonstrated that the N-terminal 220 amino acids are not important for the catalytic activity but, rather, for the stimulation by PCNA (Brown and Campbell, 1993). Detailed analysis of the interaction, using synthetic polypeptides, showed that a peptide derived from residues 129–149 specifically inhibits *in vitro* stimulation of Polδ by PCNA, suggesting that this region is involved in the interaction between the two proteins (Zhang *et al.*, 1995).

Several approaches have been taken to identify the regions on PCNA that are involved in the binding with Polδ. One approach was alanine scanning of the charge residues on the surface of PCNA. Using this technique residues of PCNA important for the interaction with Polδ have been identified in the human (Fukuda *et al.*, 1995), *S. pombe* (Arroyo *et al.*, 1996), and *S. cerevisae* (Ayyagari *et al.*, 1995) proteins. These studies have demonstrated that amino acids from different regions of PCNA play a role in the binding with Polδ. Antibody that inhibits Polδ stimulation by PCNA has also been studied and revealed that antibodies whose epitope spans the connector loop between the two domains of PCNA inhibit *in vitro* DNA replication (Roos *et al.*, 1996).

What is the structure of PCNA which is recognized by Polδ? PCNA is a trimeric ring which exerts its activity by braceleting DNA. PCNA mutants that can not form trimers fail to stimulate Polδ. A mutant form of human PCNA, with a single amino acid substitution at position 114 (Tyr replaced by Ala), causes PCNA to become a monomer (Jonsson *et al.*, 1995). This mutant cannot be assembled around DNA by RF-C and does not stimulate Polδ (Jonsson *et al.*, 1995). In *S. cerevisae* a PCNA mutation has been identified that causes PCNA to monomerize (Ayyagari *et al.*, 1995). This monomeric form of PCNA, like the human counterpart, could not support *in vitro* replication and only partial replication could be observed in the presence of crowding agents (Ayyagari *et al.*, 1995). Based on these results one can conclude that stable interaction between Polδ and PCNA is dependent upon the integrity of the trimeric ring. It is conceivable that the binding involves simultaneous interactions with more than one monomer. These experiments also suggest that the ring shape is important for the recognition and/or assembly of PCNA around DNA by RF-C (Jonsson *et al.*, 1995). Thus the failure of these mutant forms of PCNA to support DNA replication may lie in their inability to be assembled around the template.

DNA polymerase ε

A second polymerase that is stimulated by PCNA is Polε. Polε contains a 250 kDa catalytic subunit and several associated proteins (Wang, 1996 and references therein). Although studies in yeast have shown that Polε is an essential protein required for DNA replication (Morrison *et al.*, 1990; Araki *et al.*, 1992), its exact role is not yet clear. In an SV-40 *in vitro* replication assay Polε does not substitute well for Polδ resulting in short leading strand products (Lee *et al.*, 1991). These observations led to the suggestion that Polε may be involved in lagging strand synthesis. However, it has been shown that Polδ is important for the completion of the lagging strand (Waga and Stillman, 1994). Thus, the current model is that Polδ is the principle replicase and is responsible for the replication of both the leading and lagging strands. Polε, however, may still play a role in cellular DNA replication. There is clear evidence for an important role played by Polε in DNA repair. Polε was shown to be a part of the nucleotide excision repair pathway and a member of the cell cycle response to DNA damage (Navas *et al.*, 1995; Aboussekhra *et al.*, 1995 reviewed in Sancar, 1996; Wood, 1996).

In vitro studies have revealed that PCNA is important for the activity of Polε at physiological ionic strength. Although Polε is active in *in vitro* replication when assayed without salt, when the replication reaction was performed in the presence of moderate salt concentrations, PCNA was required for Polε activity (Yoder and Burgers, 1991; Lee *et al.*, 1991). The stimulation of Polε by PCNA implies an interaction between them. Direct evidence for a physical interaction between the two proteins has not yet been found. Because not all the subunits of Polε have been identified and isolated the analysis of the interactions with other protein is more complicated.

Flap endonuclease (FEN-1)

FEN-1, also called maturation factor 1 (MF-1) and DNase IV, is a 42 kDa protein with 5′ flap endonuclease activity on branched DNA molecules (flap structures) and a 5′ to 3′ exonuclease activity (Harrington and Lieber, 1994). The 5′ to 3′ exonuclease activity of FEN-1, along with the activity of RNase H, was shown to be required for Okazaki fragment maturation (Figure 2) (Ishimi *et al.*, 1988; Harrington and Lieber, 1994).

FEN-1 was shown to bind PCNA when crosslinked to a column, and association with PCNA stimulates FEN-1 activity (Li *et al.*, 1995). The stimulation could be observed only if the PCNA was encircling the DNA, regardless of whether it was loaded by RF-C or was allowed to slide onto the duplex. A mutant form of PCNA, which cannot form a trimer, can bind FEN-1 but does not stimulate its activity (Li *et al.*, 1995). These observations suggest an important role of PCNA not only in the replication of the lagging strand as a processivity factor of Polδ and Polε but also via its interaction and stimulation of FEN-1.

Gadd45

The growth arrest and DNA damage (Gadd) gene family is induced upon DNA damage (reviewed in Fornace, 1992). Gadd45 was identified by its elevated mRNA expression following DNA damage or growth arrest (Fornace *et al.*, 1988, 1989), and is a 21 kDa protein with an unknown function. It is a cell cycle-regulated nuclear protein that reaches maximal expression levels in G_1 (Kearsey *et al.*, 1995a), which may explain the growth arrest phenotype upon overexpression (Zhan *et al.*, 1994). In several cases the elevated transcription of the Gadd45 gene observed upon exposure to DNA damaging agents (e.g. ionizing irradiation) is mediated by the p53 protein (Figure 3). In other cases, however, the upregulated Gadd45 expression is in a p53 independent manner via an unclear mechanism. In several of these cases, however, where the p53 gene has been deleted or mutated, the expression levels of Gadd45 are compromised (e.g. Smith *et al.*, 1995; Graniela Sire *et al.*, 1995; Zhan *et al.*, 1996).

Gadd45 was shown to co-immunoprecipitate with PCNA (Smith *et al.*, 1994b; Hall *et al.*, 1995) and a direct interaction between the two proteins was also observed using a Far-Western technique (Chen *et al.*, 1995a). Further studies, using the two hybrid system, found that the N-terminal region of Gadd45 is involved in binding to PCNA. Three regions on PCNA have been shown to contribute to the binding (Hall *et al.*, 1995). The role played by the binding between the two proteins is not yet clear, but presumably plays a role in the cellular response to DNA damage. Gadd45 is induced upon DNA damage and PCNA was shown to be an important component for nucleotide excision repair of damaged DNA (Aboussekhra *et al.*, 1995; Shivji *et al.*, 1992; Nichols and Sancar, 1992; reviewed in Wood, 1996). Smith *et al.* (1994b) reported that the interaction of PCNA with Gadd45 stimulated nucleotide excision repair. Further studies which used cell free extracts and purified proteins have revealed that the interaction between PCNA and Gadd45 is not involved in this repair process (Kazantsev and Sancar, 1995; Kearsey *et al.*, 1995b). Further studies are needed in order to determine the function of Gadd45 in the normal cell cycle and upon DNA damage. This understanding may also shed light on the specific role(s) played by its interactions with PCNA.

Cyclin-D

Cyclin-D plays an important role in the passage through G_1 into S-phase of the cell cycle. Association between cyclin-D and different cyclin dependent kinases (cdks) affects essential steps in early G_1 (reviewed in Sherr, 1994; Hunter and Pines, 1994). One of the roles of the cyclin-D-cdk complex is to phosphorylate the retinoblastoma (Rb) protein. Rb inhibits the function of E2F, which prevents progression through G_1. Phosphorylation of Rb by the cyclin-D-cdk complex in G_1 is required to inactivate its restraining effect on cell cycle progression by, at least in part, relieving the inhibition of E2F and therefore allowing the cell cycle to proceed.

A possible interaction between PCNA and cyclin-D was suggested due to the finding that the two proteins co-immunoprecipitate in a complex which also included a 21 kDa protein (later identified as Cip1; discussed below) and Cdks (Xiong *et al.*, 1992; Zhang *et al.*, 1993). Direct binding between PCNA and cyclin-D was detected using immunoprecipitation (Matsuoka *et al.*, 1994). Deletion analysis of PCNA narrowed the binding site to the N- and C-terminal regions of PCNA. These *in vitro* studies demonstrated a direct interaction which does not require any other cellular factor (Matsuoka *et al.*, 1994).

In vivo studies of human fibroblasts shed a light on the role played by the interactions between cyclin-D and PCNA (Pagano *et al.*, 1994). In these studies different combinations of cyclins, cdks and PCNA have been expressed in fibroblast cells and analysed for their effect on DNA replication. These experiments have demonstrated that the binding of cyclin-D to PCNA sequestered PCNA and prevented PCNA-dependent replication (Pagano *et al.*, 1994). Both chromosomal and repair DNA synthesis were inhibited when cyclin-D was overexpressed. The inhibition could be relieved, however, by co-expression of PCNA (Pagano *et al.*, 1994). The interpretation of these results is that the interaction between PCNA and cyclin-D prevents premature DNA synthesis during the G_1-phase of the cell cycle. During the G_1-to-S transition there is a reduction in cyclin-D levels resulting in the release of PCNA from the complex, and leading to the initiation of chromosomal DNA replication. Similarly, upon DNA damage, cyclin-D levels decline, releasing PCNA and allowing repair DNA synthesis to occur. Whether the binding between PCNA and cyclin-D plays other roles in cell cycle progression is not yet known.

p21

The best characterized protein that interacts with PCNA but is not a part of the DNA polymerase holoenzyme is p21. p21, also called Cip1, Mda6 and Waf1, was identified as a 21 kDa protein that associated in a complex with PCNA, cyclins and cdk (Xiong *et al.*, 1992, 1993). Subsequently, the gene

encoding p21 was isolated independently by several groups using different assays. p21 was isolated by microsequencing of the 21 kDa protein present in the quaternary complex with cyclin-cdk-PCNA (Xiong *et al.*, 1993). p21 was also isolated based on its elevated transcript levels in a p53 dependent manner (Waf1: wild-type p53 activated fragment-1) (El-Deiry *et al.*, 1993), the ability of the p21 protein to interact with Cdk2 (Cip1; Cdk interacting protein-1) (Harper *et al.*, 1993), the ability to block entry into S phase and be upregulated at senescence (Sdi1: senescent cell derived inhibitor-1) (Noda *et al.*, 1994), and as a protein whose expression is induced as a function of terminal differentiation of melanoma cells (Mda6; melanoma differentiation associated gene-6) (Jiang *et al.*, 1995b). Like Gadd45, p21 transcripts are dramatically increased upon DNA damage. Although the protein was isolated based on its upregulation by p53 there are examples in which p21 expression is p53 independent (e.g. Michieli *et al.*, 1994; Parker *et al.*, 1995) (Figure 3). Several factors have been shown to be involved in p21 transcription and may play a role in the p53 independent upregulation of p21. These trans-activators include the muscle specific transcription factor MyoD (Halevy *et al.*, 1995; Parker *et al.*, 1995), the STAT transcription factor (Chin *et al.*, 1996), the enhancer binding protein C/EBPα (Timchenko *et al.*, 1996) and the receptor for Vitamin D_3 (Liu *et al.*, 1996). p21 is an inhibitor of Cdk kinase; by interacting with the cdk-cyclin complex it inhibits the kinase activity of the complex and brings about cell cycle arrest (Xiong *et al.*, 1993; Harper *et al.*, 1993; reviewed in Sherr and Roberts, 1995; Harper and Elledge, 1996). The region which interacts with Cdk was determined and it was shown that mutations that lack the ability to bind Cdk exhibit a reduction in inhibition of DNA synthesis and cell growth (Chen *et al.*, 1995b; Nakanishi *et al.*, 1995a,b).

In vitro studies have shown that p21 inhibits processive DNA synthesis and that the inhibition depends on PCNA (Flores-Rozas *et al.*, 1994; Waga *et al.*, 1994). The inhibition of DNA replication by p21 in a PCNA dependent manner, and the identification of the two proteins in the same complex, suggests an interaction between the two. p21 directly binds PCNA. The binding has been documented using immunoprecipitation, gel-filtration, glycerol gradient sedimentation and surface plasmon resonance (Flores-Rozas *et al.*, 1994; Waga *et al.*, 1994), and was shown to have a K_d of 2.5 nM (Gibbs *et al.*, 1997). Thus, p21 has two distinct inhibitory effects on the entry of the cell into S-phase. One is to inhibit the kinase activity of Cdk and the other is to inhibit DNA replication via an interaction with PCNA (Figure 2). These two distinct inhibitory effects reside in different domains of the protein (Chen *et al.*, 1995b; Luo *et al.*, 1995; Goubin and Ducommun, 1995). The N-terminal domain of p21 contains the Cdk inhibitory activity (Chen *et al.*, 1995b; Goubin and Ducommun, 1995; Harper *et al.*, 1995; Luo *et al.*, 1995; Nakanishi *et al.*, 1995a). The C-terminal domain of p21 is involved in the binding of PCNA (Chen *et al.*, 1995b, 1996; Goubin and Ducommun, 1995; Luo *et al.*, 1995; Nakanishi *et al.*, 1995b; Warbrick *et al.*, 1995). The N-terminal half of PCNA participates in the binding to p21 (Chen *et al.*, 1996).

The existence of two distinct domains which are involved in the interaction of p21 with PCNA and cdk suggests the possibility that p21 serves as a bridge between the cyclin-cdk complex and PCNA to bring about the tertiary complex. Support for this idea comes from the failure, so far, to demonstrate a direct interaction between cdk and PCNA (unpublished observation). Complexes involving cyclin-D, however, may be mediated, at least in part, by cyclin-D, since a direct interaction between this factor and PCNA has been demonstrated (see above).

It was demonstrated that p21 inhibits DNA replication *in vitro*. Further studies have demonstrated that a short peptide derived from the C-terminus of p21 is sufficient for the inhibition both *in vitro* (Warbrick *et al.*, 1995) and *in vivo* (Chen *et al.*, 1996). What is the mechanism by which p21 operates to inhibit DNA replication? Early studies, using PCNA cross-linking experiments in the presence of p21, suggested that p21 might dissociate the PCNA trimer and thus prevent the formation of the clamp around DNA (Chen *et al.*, 1995a). This would prevent processive DNA synthesis, which depends upon PCNA (described above). Recently, a functional assay was used to analyse the inhibition process. It was demonstrated that p21 does not substantially inhibit the assembly of PCNA around DNA by RF-C (Podust *et al.*, 1995; Gibbs *et al.*, 1997). The ability of PCNA to be assembled around DNA in the presence of p21 strongly suggests that the interaction between the proteins does not affect the integrity of the ring structure or the interaction between PCNA and RF-C. Further support for this notion comes from the recently solved three-dimensional structure of human PCNA complex with a peptide derived from the last 22 amino acids of p21 (Gulbis *et al.*, 1996). In the co-crystals, the PCNA molecule remains as a trimer where each monomer interacts with one p21 peptide leading to a 3:1 ratio of peptide to PCNA timer, which is in agreement with the surface plasmon resonance results (Flores-Rozas *et al.*, 1994). Moreover, in the three-dimensional structure the central cavity of the ring, where DNA is inserted, stays open and is not blocked by the peptide. Based on these studies it became apparent that the inhibition of processive replication by the binding of p21 to PCNA is not via the inhibition of the loading onto DNA by RF-C and/or the structure of the ring.

Thus, p21 might prevent the interaction between PCNA and Polδ. Although this is currently under investigation, there are several lines of evidence supporting this possibility. Each PCNA monomer is composed of two globular domains linked together by a connector loop (Krishna *et al.*, 1994; Gulbis *et al.*, 1996). The three-dimensional structure shows that the p21 peptide contacts three regions of the PCNA surface. If forms contacts with residues from both domains and with the connector between the domains (Gulbis *et al.*, 1996). It was recently shown that antibody whose epitope spans the connector loop inhibits *in vitro* DNA replication (Roos *et al.*, 1996). Also, a substitution of Ala instead of Asp at residue 122 of the connecting loop (the human connector loop spans residues 119-133) was shown to affect the stimulation of Polδ by PCNA (Fukuda *et al.*, 1995).

PCNA is also required for nucleotide excision repair (Shivji *et al.*, 1992). Does the interaction between p21 and PCNA inhibit DNA repair? Different studies addressing

these questions yielded conflicting results. Several groups reported that p21 does not inhibit PCNA-dependent nucleotide excision repair (Shivji *et al.*, 1994; Li *et al.*, 1994, 1996). Others, however, reported that p21 inhibits both DNA excision repair (Pan *et al.*, 1995) and mismatch repair (Umar *et al.*, 1996). The reason for the discrepancy is unclear. Several explanations, however, may account for the differences. One of the studies was done *in vivo* (Li *et al.*, 1996) and thus makes comparison to *in vitro* studies rather difficult. The cell extracts used for the *in vitro* studies might have some differences in protein composition as a result of cell lines and/or purification protocols, and different assays and conditions have been used by the different laboratories. It is important to note that the *in vivo* data show that as a response to DNA damage there is an inhibition of chromosomal replication but not of DNA repair; this is consistent with the studies that demonstrated no effect of p21 on DNA repair. As described above, Polε plays a role in DNA repair, but it is not yet clear whether PCNA-p21 interactions inhibit the binding between Polε and PCNA. Thus, if the interactions with p21 inhibit the binding of PCNA to Polδ but not to Polε then chromsomal DNA replication will stall upon DNA damage, but the repair pathways will not be affected. Further studies are needed in order to establish the precise role of p21-PCNA interactions in DNA repair processes.

Interestingly, p21 was also shown to interact with Gadd45 in a co-immunoprecipitation assay (Kearsey *et al.*, 1995a). Detailed analysis of the binding suggests that Gadd45 binds both the N and the C-terminus of p21. The C-terminus of p21 is also involved in the interaction with PCNA. This may explain the observation that Gadd45 and p21 compete in the binding of PCNA (Chen *et al.*, 1995a).

Concluding remarks

Since PCNA was first identified almost 20 years ago a vast amount of information has accumulated on its structure and function. The three dimensional structure of PCNA from yeast and human has been determined and the role of PCNA in DNA replication, as a processivity factor for the Polδ holoenzyme, has been established. In recent years it has also become apparent that PCNA has additional functions in the cell in DNA repair and perhaps in transcription and cell cycle regulation.

Although the role of PCNA in DNA replication is well understood, the mechanism by which PCNA is assembled around the primer by the RF-C complex is largely unknown. The recent purification of all five components of RF-C (Cullmann *et al.*, 1995 and references therein) should provide the tools to study the loading mechanism.

The determination of the three dimensional structure of the protein allows a new strategy for its study. The three-dimensional structure can be used to perform structure/function studies and to analyse the role of particular regions of PCNA in its interactions with other proteins. Only a few of these interactions have been analysed to date at the molecular level. The structure of the protein also helped to explain several of the phenotypes associated with mutations within PCNA genes such as cold sensitivity and mutations that affect the activity of PCNA, for example mutations in the interfaces between the monomers within the trimer. Altering specific regions of the protein may shed a light on their relative importance for the diverse function of PCNA.

Is PCNA transcription regulated by p53 and why? Although p53 can regulate the transcription of a reporter gene fused to the PCNA promoter it remains to be determined whether PCNA is regulated in a p53-dependent manner within the cell.

Recently, several questions about different aspects of PCNA function have begun to be explored. What are the roles played by the interactions between PCNA and the proteins involved in cell cycle progression and regulation? What are the functions of the interactions with Gadd45 and p21? Do these proteins bind PCNA during DNA repair and perhaps prevent chromosomal replication? Future studies are needed to answer these and other questions including the importance of the binding of PCNA to different cellular components. Furthermore, the list of proteins that interact with PCNA might not be exhausted and new proteins might be identified in the future. One might also try to explore the possibility that PCNA is involved in gene transcription. Although to date only viruses utilize the processivity factors of DNA polymerases as a part of the transcription machinery it might also generalize to the involvement of PCNA in the expression of cellular genes.

PCNA is a fascinating protein because of its unique ring-shaped structure and its diverse functions within the cell. Although much has been studied about it there is still long way to go until we fully appreciate its important role in all living cells.

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WAF1, a Potential Mediator of p53 Tumor Suppression

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Summary

The ability of p53 to activate transcription from specific sequences suggests that genes induced by p53 may mediate its biological role as a tumor suppressor. Using a subtractive hybridization approach, we identified a gene, named *WAF1*, whose induction was associated with wild-type but not mutant p53 gene expression in a human brain tumor cell line. The *WAF1* gene was localized to chromosome 6p21.2, and its sequence, structure, and activation by p53 was conserved in rodents. Introduction of *WAF1* cDNA suppressed the growth of human brain, lung, and colon tumor cells in culture. Using a yeast enhancer trap, a p53-binding site was identified 2.4 kb upstream of *WAF1* coding sequences. The *WAF1* promoter, including this p53-binding site, conferred p53-dependent inducibility upon a heterologous reporter gene. These studies define a gene whose expression is directly induced by p53 and that could be an important mediator of p53-dependent tumor growth suppression.

Introduction

Inactivation of p53 is a common event in the development of human neoplasia (Hollstein et al., 1991). A variety of mechanisms can lead to such functional inactivation, including missense mutation (Baker et al., 1989) and interaction with oncogenic viral or cellular proteins (Mietz et al., 1992; Momand et al., 1992). Wild-type p53 has been shown to be a suppressor of tumor cell growth (for reviews see Mercer, 1992; Oren, 1992; Lane, 1992; Perry and Levine, 1993). Inactivation of p53 by any of the above mechanisms thereby leads to a selective growth advantage, generally observed as tumor progression.

The mechanism underlying p53 growth suppression is still undefined. Several biochemical features of p53 have been elucidated, and at least two of these are currently of much interest. First, p53 has been shown to suppress a variety of promoters containing TATA elements (e.g., Ginsberg et al., 1991; Santhanam et al., 1991; Kley et al., 1992; Mack et al., 1993). This suppression is apparently sequence independent and may involve p53 binding to the TATA-binding protein or to other transcription factors (Seto et al., 1992; Truant et al., 1993; Ragimov et al., 1993; Martin et al., 1993; Liu et al., 1993). Second, p53 can bind to DNA in a sequence-specific manner (Kern et al., 1991). A 20 bp consensus-binding site, consisting of two copies of the 10 bp sequence 5′-PuPuPuC(A/T)(T/A)GPyPyPy-3′, separated by up to 13 bp, has been identified (El-Deiry et al., 1992; Funk et al., 1992). Both copies of the 10 bp sequence are required for efficient binding by p53. p53 contains a strong transcriptional activation sequence near its amino terminus (Fields and Jang, 1990; Raycroft et al., 1990) and can stimulate the expression of genes downstream of its binding site. Such stimulation has been demonstrated in both mammalian (Kern et al., 1992; Funk et al., 1992; Zambetti et al., 1992) and yeast cells (Scharer and Iggo, 1992; Kern et al., 1992), as well as in an in vitro system (Farmer et al., 1992).

The sequence-specific transcriptional activation by p53 has led to the hypothesis that p53-induced genes may mediate its biological role as a tumor suppressor (Vogelstein and Kinzler, 1992). To date, several genes containing p53-binding sites have been identified. These include muscle creatine kinase (Weintraub et al., 1991; Zambetti et al., 1992), *GADD45* (Kastan et al., 1992), *MDM2* (Barak et al., 1993; Wu et al., 1993), and a *GLN* retroviral element (Zauberman et al., 1993). Each of these genes contains a 20 bp sequence with high homology to the p53 consensus-binding site (Prives and Manfredi, 1993). The p53-binding sites in *GADD45* and *MDM2* are located within introns, the muscle creatine kinase site is 3 kb upstream of the transcription start site, and the *GLN* element is located within a long terminal repeat. The relationship of any of these genes to suppression of cell growth by p53 remains unclear. It has been suggested that *MDM2* may be a feedback regulator of p53 action by being transcriptionally induced (Barak et al., 1993; Wu et al., 1993) and then inhibiting p53 function (Momand et al., 1992; Oliner et al., 1993; Wu et al., 1993). In this regard, *MDM2* functions as an oncogene rather than as a tumor suppressor gene (Fakharzadeh et al., 1991; Finlay, 1993).

In an effort to identify biologically important genes that are transcriptionally regulated by p53, we constructed a cDNA library enriched for the presence of such genes. Using a subtractive hybridization technique, we identified a highly induced gene, named wild-type p53-activated fragment 1 (*WAF1*). We showed that *WAF1* is directly regulated by p53 and can itself suppress tumor cell growth in culture. Thus, *WAF1* may be an important component of the p53 growth suppression pathway.

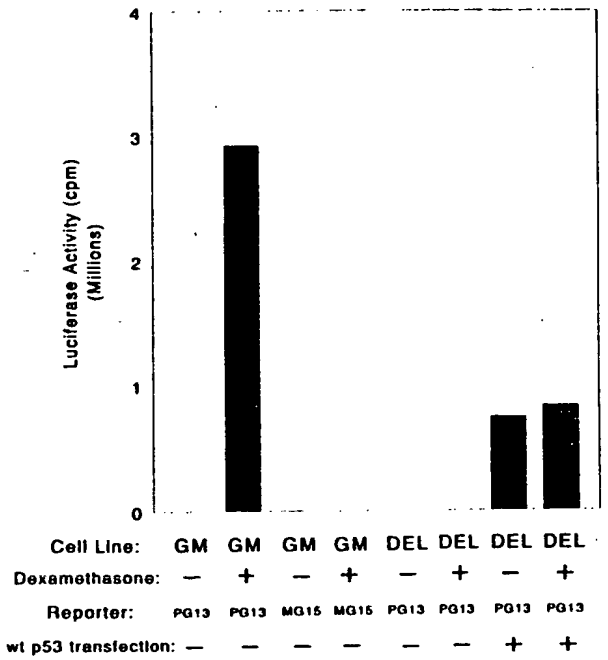

Figure 1. p53-Dependent Transactivation in GM and DEL Cell Lines

GM cells (containing inducible wild-type p53) or DEL cells (containing inducible mutant p53) were transfected with reporter plasmids as indicated, and luciferase activity was measured after 18 hr in the absence (minus) or presence (plus) of dexamethasone. Wild-type (wt) p53 expression plasmid was cotransfected with PG13–Luc into DEL cells in the two lanes at the right.

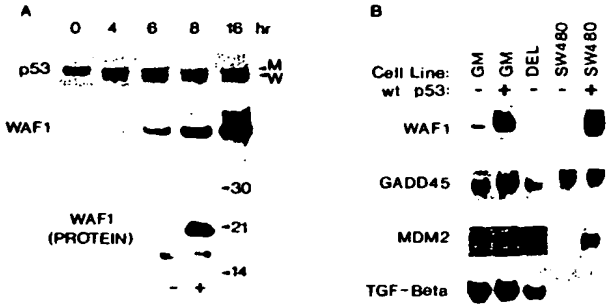

Figure 2. Induction of *WAF1* by p53

(A) A Northern blot was prepared using 10 μg of total RNA isolated from GM cells treated with dexamethasone for 0–16 hr and probed with p53 cDNA or *WAF1* cDNA. The endogenous mutant (m) and induced wild-type (w) p53 mRNA species are indicated. Lysates from GM cells induced with dexamethasone for 22 hr (plus) or uninduced (minus) were used in Western blot analysis with anti-WAF1 antisera.

(B) A Northern blot was prepared from RNA of GM cells in the absence or presence of dexamethasone for 16 hr (lanes 1 and 2, respectively), from DEL cells treated with dexamethasone for 16 hr (lane 3), or from SW480 cells infected with Ad-gal (lane 4) or Ad-p53 (lane 5) for 16 hr. The blot was probed with *WAF1* DNA, *GADD45* DNA, *MDM2* DNA, or TGFβ DNA, as indicated.

Results

Definition of a p53-Responsive System

As a first step toward the isolation of p53-regulated genes, we determined optimal cell culture conditions under which an exogenous wild-type p53 protein could activate transcription through specific DNA binding. A reporter plasmid containing a p53 DNA-binding site upstream of a basal promoter (Kern et al., 1992) linked to a luciferase reporter gene (PG13–Luc) was cloned and cotransfected into SW480 colon cancer cells with either a human wild-type p53 expression plasmid (p53-wt) or a mutant p53 expression plasmid (p53-273). High luciferase activity was observed only when wild-type p53 was present (data not shown). No luciferase activity was detected if the reporter plasmid contained mutant p53-binding sites (MG15–Luc), regardless of whether or not wild-type p53 was present. This validated reporter was then used in a p53-inducible system. The glioblastoma cell line GM contains an endogenous mutant p53 gene (Ullrich et al., 1992) and exogenous wild-type p53 under the control of a steroid responsive promoter. A moderate amount of wild-type p53 is induced in these cells by dexamethasone (Mercer et al., 1990). The related line DEL expresses the same endogenous mutant p53 and an additional dexamethasone-inducible mutant p53 (Lin et al., 1992). Both cell lines were transfected with either PG13–Luc or MG15–Luc and incubated in the presence or absence of dexamethasone. Figure 1 shows that dexamethasone-induced wild-type p53 (GM) but not mutant p53 (DEL) expression activated the luciferase reporter. No luciferase activity was observed when the p53-binding site was mutant (MG15–Luc) or when the p53 protein was mutant (GM without dexamethasone or DEL with or without dexamethasone). Transfection of wild-type p53 into DEL cells activated the PG13–Luc reporter with or without dexamethasone (Figure 1), confirming that the failure of expression of luciferase reporter gene in this cell line was due to the absence of wild-type p53. These experiments demonstrated that reporter gene expression in these two cell lines was dependent on the presence of wild-type p53.

Subtractive Hybridization

Based on the reporter gene experiments, we chose to use subtractive hybridization to identify endogenous genes regulated by p53 in GM cells. To determine the optimal time to isolate RNA enriched for p53-induced genes, Northern blot analysis was performed, using RNA isolated from GM cells at various intervals following dexamethasone induction. Figure 2 shows that under the logarithmic growth conditions used, the exogenous wild-type p53 mRNA was detectable by 4 hr after induction and remained elevated for at least 16 hr in GM cells upon dexamethasone induction. A p53-induced cDNA library was therefore prepared from GM cells treated with dexamethasone for 6 hr (see Experimental Procedures). Of the clones obtained, 80% carried inserts, generally of 1.5–2.0 kb in length. A total of 120,000 clones were screened by hybridization to a subtracted p53-induced cDNA probe. This probe was made from cDNA of dexamethasone-induced GM cells after subtraction with an excess of dexamethasone-induced DEL RNA. Control experiments showed that the subtraction procedure used, involving chemical cross-

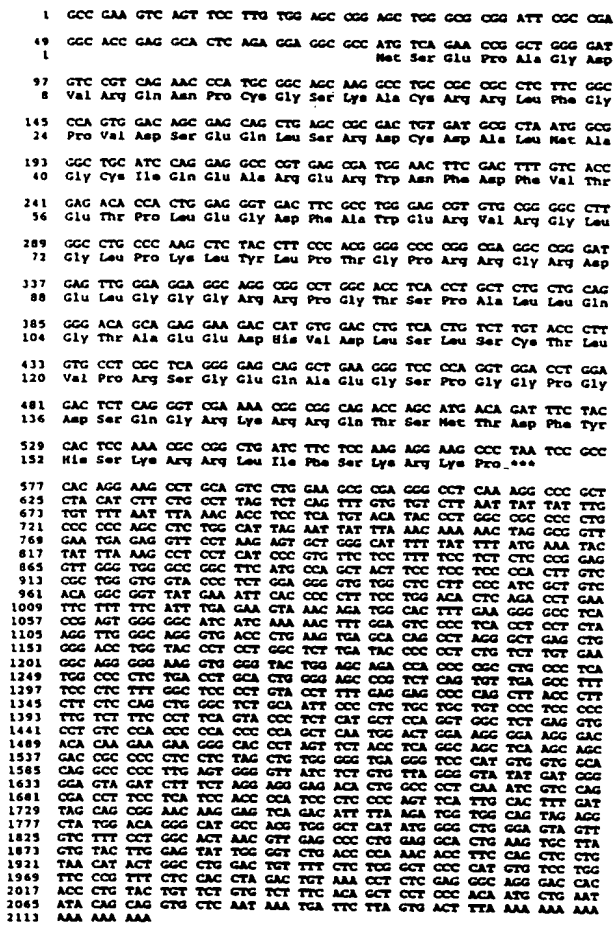

Figure 3. cDNA and Predicted Amino Acid Sequence of Human *WAF1*

The predicted translation begins at nucleotide 76 and ends at nucleotide 567.

linking (Hampson et al., 1992), provided an enrichment of over 100-fold for cDNA sequences not present in the RNA used for subtraction (data not shown). Following hybridization to the subtracted probe, the clones were rehybridized to a probe made from RNA of dexamethasone-induced DEL cells. A total of 99 clones differentially hybridized to the subtracted probe on the initial screen, and 45 of these reproducibly displayed differential hybridization when retested. Hybridization probes were prepared from these clones and used in Northern blots containing RNA isolated from dexamethasone-treated or untreated GM cells. Of the 45 clones, 28 were found to be highly induced upon dexamethasone treatment. The other 17 clones were less robustly induced by dexamethasone and were not studied further. Hybridization, sequencing, and restriction endonuclease analysis indicated that all of the 28 highly induced cDNA clones were derived from a single 2.1 kb mRNA. The gene encoding this message was named *WAF1*. Hybridization of individual *WAF1* clones to the cDNA library revealed that *WAF1* cDNA was present at a frequency of 0.4% following dexamethasone induction.

Structural Analysis of *WAF1*

Of the 28 *WAF1* clones, 18 appeared to contain near full-length cDNA, predicted to be 2.1 kb on the basis of Northern blot analysis (Figure 2A). DNA sequencing revealed that most of the clones contained the same 5′ end. Because the cDNA library used was not amplified, this likely represented the 5′ end of the transcript. The *WAF1* cDNA sequence is shown in Figure 3. The first ATG codon occurred at nucleotide 76, and an in-frame termination codon occurred at nucleotide 570, predicting a translation product of 18.1 kd. In vitro transcription and translation of *WAF1* cDNA clones produced a protein of the expected size (data not shown). Additionally, GM cells induced with dexamethasone produced a protein of 21 kd reactive with anti-WAF1 antibodies (see Figure 2A). These antibodies localized WAF1 protein to the nucleus of dexamethasone-induced GM cells (W. S. E.-D., B. V., M. Burrell, and D. Hill, unpublished data). Analysis of the amino acid sequence of WAF1 protein revealed a cysteine-rich region $C(X)_4C(X)_{15}C(X)_6C$ between amino acids 13 and 41 with the potential for zinc binding (Berg, 1986) as well as a basic region between amino acids 140 and 163 containing two potential bipartite nuclear localization signals (Robbins et al., 1991) near the carboxyl terminus. No significant homologies at the amino acid level were found to known proteins (National Biomedical Research Foundation PIR release #35.0). Southern blot analysis showed that *WAF1* was probably a single copy gene, with no close relatives in the human genome (data not shown).

To identify the chromosomal location of the *WAF1* gene, a human genomic *P1* clone (*P1-WAF1*) containing *WAF1* sequences was obtained (see Experimental Procedures). The clone contained about 85 kb of DNA, and partial sequencing revealed that the *WAF1* gene consisted of three exons of 68, 450, and 1600 bp (exons 1, 2, and 3, respectively). The translation initiation signal was contained in exon 2, a relatively long coding exon (Sterner and Berget, 1993). The *P1-WAF1* clone was labeled with biotin and hybridized to metaphase chromosomes as previously described (Meltzer et al., 1992). A total of 18 metaphase cells were examined, and each had at least one double fluorescent signal (i.e., signals on each of two chromatids) on the middle of the short arm of chromosome 6. In 15 of 18 cells, double signals were observed on both chromosome 6 homologs. Only chromosomes in which both chromatids displayed a signal were included for analysis, making the background hybridization close to zero. The same cells subjected to fluorescence in situ hybridization had been previously G-banded using trypsin–Giemsa and photographed to allow direct comparison of the results. The results demonstrated that sequences hybridizing to *WAF1* DNA fragment were localized to 6p21.2.

Induction of *WAF1*

If *WAF1* is important for p53 function, one might expect that it would be induced in more than one human cell type following wild-type p53 expression; that it would be highly conserved among species, because p53 is conserved both functionally and structurally; and that its induction by p53

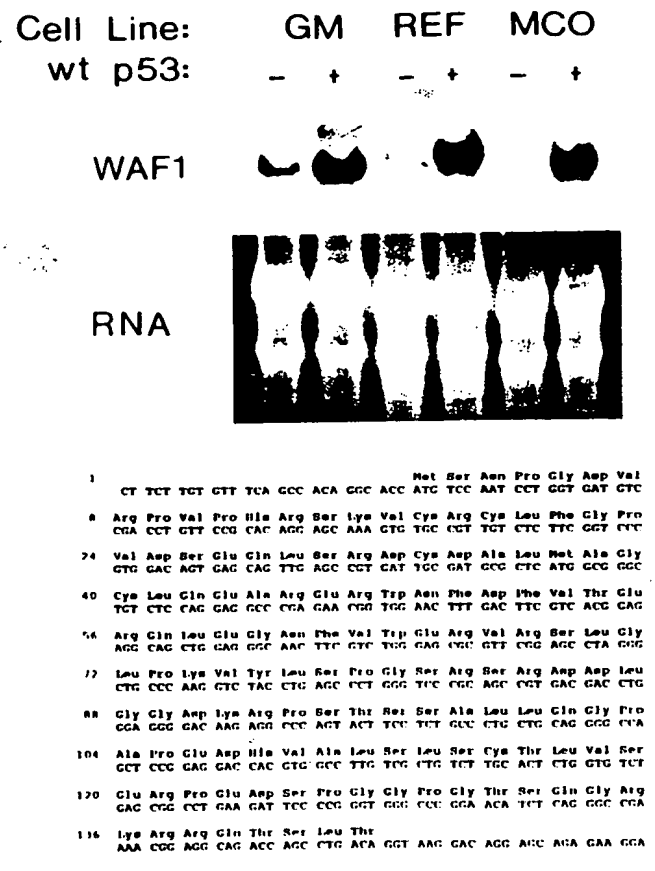

Figure 4. *WAF1* Induction by p53 Is Conserved in Rat and Mouse

A Northern blot was prepared using RNA from GM cells, untreated (lane 1) or treated for 6 hr with dexamethasone (lane 2), from REF-112 cells grown at 37°C (uninduced; lane 3) or 31°C (lane 4), or from MCO1 cells infected with Ad-gal (lane 5) or Ad-p53 (lane 6). The RNA was hybridized with a human (lanes 1 and 2) or mouse (lanes 3–6) *WAF1* probe. An ethidium bromide stain of the gel prior to transfer is also shown. The nucleotide and predicted amino acid sequence of the mouse *WAF1* second exon is shown at the bottom.

would also extend across species. These predictions were tested in the following series of experiments.

Figure 2B illustrates the expression of *WAF1* in GM cells following dexamethasone treatment for 16 hr (lane 2), compared with either uninduced GM cells (lane 1) or dexamethasone-treated DEL cells containing induced mutant p53 (lane 3). Controls for the experiment included two other genes known to be induced by p53, *MDM2* and *GADD45*, as well as an unrelated gene, transforming growth factor β (TGFβ). Both *MDM2* and *GADD45* were induced in GM cells when wild-type p53 was present, but less so than *WAF1* (see Figure 2B).

To examine the induction of *WAF1* by p53 in a different human cell line, a wild-type p53 construct in an adenoviral vector (Ad-p53) was used to infect SW480 colon cancer cells. That Ad-p53 produced transcriptionally active p53 was demonstrated by assaying an SW480 cell line carrying a stably integrated reporter responsive to wild-type but not mutant p53 (see Experimental Procedures). SW480 cells were infected with either Ad-p53 or Ad-gal (a control adenoviral vector producing β-galactosidase instead of p53) for 16 hr. *WAF1* mRNA was highly induced in SW480 cells infected with Ad-p53 (see Figure 2B, lane 5), but not those infected with Ad-gal (lane 4).

We next assessed the evolutionary conservation of *WAF1*. So-called zoo blots revealed that single copy sequences from mouse and rat cells hybridized to the human *WAF1* clone, and we obtained a clone containing the *WAF1* gene by screening a mouse genomic library. The nucleotide and predicted amino acid sequence of the mouse *WAF1* second exon is shown in Figure 4. The mouse and human *WAF1* second exon sequences were 75% identical and 79% similar at the amino acid level. A stretch of 26 amino acids (human amino acids 21–56) was almost perfectly conserved, as was the zinc finger–like motif between amino acids 13 and 41 in human WAF1 ($H(X)_4C(X)_{15}C(X)_8C$ in the mouse). The positions of introns surrounding exon 2 in the *WAF1* gene were identical in human and mouse.

To determine whether rodent *WAF1* gene expression was induced by wild-type p53, two experimental systems were used. The first consisted of rat embryo fibroblasts containing a stably integrated murine temperature-sensitive mutant p53 (REF-112 cells; Michalovitz et al., 1990). These cells were transfected with the PG13–Luc reporter and incubated either at 37°C (mutant p53 conformation) or 31°C (wild-type p53 conformation) for 24 hr. No measurable increase in luciferase activity was observed at 37°C, but luciferase activity increased 1000-fold at 31°C, confirming the presence of transcriptionally active murine wild-type p53 at the latter temperature. RNA was then prepared from REF-112 cells incubated for 14 hr either at 37°C or 31°C. Figure 4 shows that expression of *WAF1* mRNA was detected at 31°C but not at 37°C, demonstrating that the *WAF1* gene is conserved in rat and that the gene is inducible by the murine p53 at the wild-type permissive temperature.

Second, the murine fibrosarcoma cell line MCO1 (Halevy et al., 1991), which lacks p53 owing to a splice site mutation and a deletion, was infected with either Ad-p53 or Ad-gal. At 22 hr following adenoviral infection, RNA was prepared and used in Northern blot analysis. Figure 4 shows that mouse *WAF1* was highly induced in MCO1 cells infected with Ad-p53, but not in cells infected with Ad-gal. Thus, *WAF1* induction by p53 was conserved in both rat and mouse cells.

WAF1 Suppresses Tumor Cell Growth

If *WAF1* plays a role in mediating the tumor growth inhibition of p53, one might expect it to have a growth suppressive role of its own. To address this possibility, mammalian expression vectors containing p53 cDNA or *WAF1* cDNA in either the sense (pC-WAF1-S) or antisense (pC-WAF1-AS) orientation were constructed. The vectors each contained a gene conferring hygromycin resistance in addition to the cDNA. The vectors were transfected into SW480 cells previously shown to be inhibited by wild-type p53 expression (Baker et al., 1990). Following transfection, cells were grown in the presence of hygromycin, and the number of colonies was scored after 2–3 weeks. The

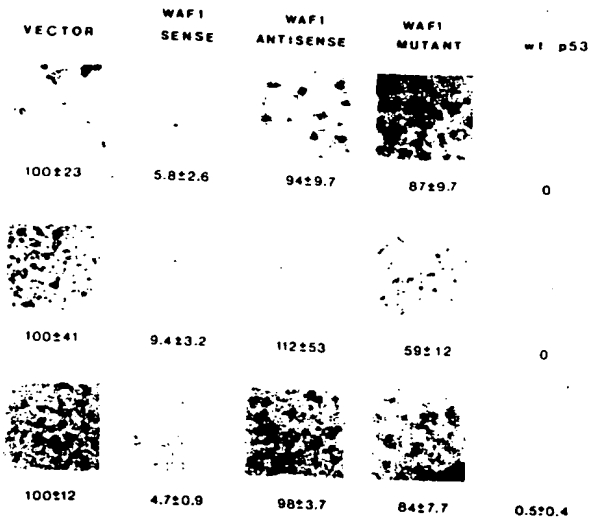

Figure 5. *WAF1* Suppresses the Growth of Human Tumor Cells

The human brain tumor line DEL (top), the human colon tumor line SW480 (middle), or the lung adenocarcinoma line H1299 (bottom) were transfected with the pCEP4 vector or with vectors encoding sense *WAF1*, antisense *WAF1*, mutant *WAF1*, or wild-type (wt) p53. The photographs show low power views of the transfected flasks following 17 days of hygromycin selection. Below each photograph, the fraction of colonies (in percent) in each flask compared with the vector transfected cells is indicated (mean of three flasks ± SD). The vector transfectants contained an average of 310, 850, and 427 colonies, respectively.

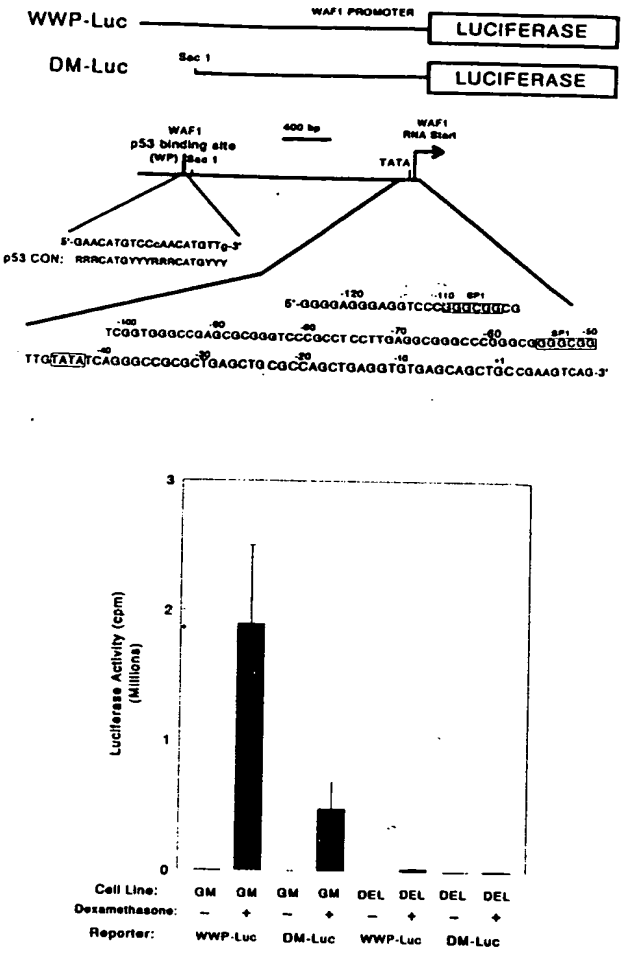

Figure 6. *WAF1* Transcriptional Regulatory Region

The diagram shows the promoter–reporter constructs and partial DNA sequence of the *WAF1* promoter. The consensus p53-binding site is compared with the p53-binding site (WP) 2.4 kb upstream of *WAF1*. The TATA element and Sp1 recognition sequences within the *WAF1* promoter are indicated by boxes. GM or DEL cells were transfected with the WWP–Luc or DM–Luc reporters (bottom), and luciferase activity was measured after incubation with or without dexamethasone for 14 hr.

data in Figure 5 show that introduction of *WAF1* sense cDNA expression vectors resulted in substantial growth suppression, as seen by a 10- to 20-fold decrease in the number of hygromycin-resistant colonies. This growth suppression was similar to, but not as complete as, that observed with p53 (Figure 5). Introduction of the *WAF1* antisense cDNA expression vector or of the vector devoid of *WAF1* sequences resulted in a similar number of clones. The few clones that did appear after transfection of the *WAF1* sense cDNA expression vector generally grew at a slow rate and were not easily passaged. Similar results were obtained in four separate experiments, each with triplicate transfections, using different preparations of plasmid DNA. Additionally, we used the brain tumor cell lines GM and DEL and the lung adenocarcinoma line H1299 in similar experiments and found that their growth was also suppressed by the introduction of wild-type *WAF1* (Figure 5; data not shown). As an additional control, we constructed a *WAF1* mutant (pC-WAF1-ES) with a stop codon at nucleotide 222. Introduction of pC-WAF1-ES into either SW480, H1299, or DEL cells did not result in significant growth suppression (Figure 5).

p53 Activation of the *WAF1* Promoter

Having demonstrated that *WAF1* expression is induced by wild-type p53, we attempted to determine whether this resulted from a direct interaction of p53 with regulatory elements in *WAF1*. To search for sequences transcriptionally responsive to p53, we used the genomic clone *P1-WAF1* in a yeast enhancer trap system. In this system, yeast cells auxotrophic for histidine were transformed with a plasmid library constructed by insertion of random fragments of *P1-WAF1* upstream of a truncated *GAL1* promoter regulating *HIS3* reporter gene expression. Clones were selected for histidine prototrophy in the presence of human p53 expression. Three libraries were constructed, using AluI, HaeIII, or Sau3AI fragments of *P1-WAF1*. Through the screening of 1.6×10^5 transformants, 22 wild-type p53-dependent histidine prototrophs were obtained. No histidine prototrophy was observed if yeast expressed mutant instead of wild-type p53. All but 1 of the 22 clones were found to contain either of two sequence elements, both matching the previously defined p53-binding site consensus. Mapping revealed that one of them was located 2.4 kb upstream of *WAF1* coding sequences (Figure 6); the other was more than 8 kb upstream (T. Waldman and W. S. E.-D., unpublished data) and was not studied further.

The yeast experiments showed that at least one p53-binding site was present near *WAF1*, and this element, when placed in an artificial system with a foreign promoter, could stimulate expression of a reporter gene in the presence of wild-type p53. To determine whether the natural promoter elements of *WAF1* could mediate p53-dependent transcriptional activation, a 2.4 kb genomic fragment, with its 3' end at nucleotide 11 of *WAF1* cDNA, was cloned upstream of a promoterless luciferase reporter gene. A partial sequence of the *WAF1* promoter and a map of this clone are shown in Figure 6. This promoter was G:C rich and contained a TATA element 43 nt upstream of the putative transcription start site. Two Sp1-binding sites were located at nucleotides −50 and −104, and there was a sequence weakly matching the p53-binding site consensus at nucleotide −75. The p53 responsive element identified in the yeast experiments was 2.4 kb upstream of *WAF1*.

Figure 6 shows that the *WAF1* promoter construct WWP–Luc activated expression of luciferase only in the presence of wild-type p53. In the absence of wild-type p53 (GM cells without dexamethasone or DEL with or without dexamethasone), expression of this reporter was less than 3% of levels observed in the presence of wild-type p53. When the 2.4 kb upstream p53-binding site was deleted (DM–Luc), the majority of the luciferase activity was abolished, though the residual activity was still wild-type p53 dependent. This observation suggests the presence of a second (weaker) p53 response element within the *WAF1* promoter, perhaps at nucleotide −75 (Figure 6).

Discussion

One of the goals of tumor biology is to unravel the pathways leading to growth suppression. For the tumor suppressor p53, a clue to the pathway was provided when it was found that p53 can bind to DNA in a sequence-specific manner and activate transcription from adjacent genes (see Introduction). This suggests that genes whose expression is activated by p53 might be mediators of p53 action (Vogelstein and Kinzler, 1992). The data described here show that *WAF1* may represent such a gene. *WAF1* expression was induced by p53, and this induction was observed in cell lines from human, mouse, and rat. Not only are the coding sequences and exon structure of *WAF1* conserved, but also its regulation by p53. This is consistent with the fact that p53 tumor suppressive function is also conserved between rodents and humans and the expectation that the mechanism of this suppression would be similarly conserved. The activation of a gene following wild-type p53 expression could be indirect, a result of induction by a second gene primarily controlled by p53. In the case of *WAF1*, the p53 induction was likely to be direct, as at least one strong functionally active binding site existed within its transcription regulatory region. The binding site functioned in yeast as well as in mammalian cells. Finally, *WAF1* could itself mimic the growth suppression of p53 when introduced into four different tumor cell lines.

Although all these experiments suggest that *WAF1* plays an important role in the p53 pathway, the results should be interpreted cautiously. First, we do not know whether WAF1-mediated growth inhibition results from the induction of apoptosis (Shaw et al., 1992; Lowe et al., 1993; Clarke et al., 1993) or of G1 arrest (Kastan et al., 1991; Lane, 1992). Second, we cannot be sure that *WAF1* is a critical target for p53. It is conceivable that the p53 DNA-binding site near the *WAF1* promoter is coincidental and that the growth inhibition mediated by *WAF1* results from an entirely separate pathway. Third, even if *WAF1* is a critical target, it may not be the only critical target. It may be part of a genetic program of growth arrest mediated by wild-type p53, and p53 may induce several downstream effectors, each with the potential to play a role in growth inhibition in some cells under certain circumstances. In this regard, we have noted in preliminary experiments that DNA damage induced by ultraviolet radiation (known to induce p53 expression; Maltzman and Czyzyk, 1984; Zhan et al., 1993) induces *WAF1* expression, but at lower levels than those observed in GM cells induced to express wild-type p53 with dexamethasone. Perhaps several growth arrest pathways exist, depending on the type of cell and its environment, as suggested by other experiments (Livingstone et al., 1992; Yin et al., 1992; Lowe et al., 1993; Clarke et al., 1993; Sherley, 1991; Zhan et al., 1993). We also note that G1 arrest induced in GM cells by mimosine or serum starvation, in the absence of wild-type p53, did not induce *WAF1* gene expression (W. S. E.-D. and B. V., unpublished data).

In the future, some of the above issues can be tested in *WAF1* mutagenesis and "knock out" experiments. A subset of the effects of p53 would be predicted to be *WAF1* dependent, assuming *WAF1* function was not redundant with other downstream genes. A detailed analysis of the cell cycle in stable cell lines carrying an inducible *WAF1* transgene may similarly provide clues as to its function in p53-mediated growth inhibition. Additionally, some tumors without p53 mutation might contain mutations of *WAF1*. Several tumors have been noted to have losses of the chromosomal region (6p21) containing *WAF1* (Solomon et al., 1991; Sato et al., 1991; Cliby et al., 1993; Lukeis et al., 1990; Morita et al., 1991; Vogelstein et al., 1989), consistent with the idea that a tumor suppressor gene resides in this area. Finally, identification of *WAF1* and its regulatory region potentially provides a novel drug discovery approach: compounds that activate expression of *WAF1* might bypass the p53 defect in tumors with endogenous p53 mutation.

After acceptance of this manuscript for publication, we learned that Harper et al. (1993 [this issue of *Cell*]) have identified a gene called *CIP1* whose product binds to cyclin complexes and inhibits the function of cyclin-dependent kinases. The sequence of *CIP1* is identical to that of *WAF1*. These results provide a dramatic example of the interplay between tumor suppressor genes and the cell cycle. In particular, the combined data suggest the following model for p53 function: p53 is not required for normal development, but in certain cellular environments (DNA damage, cellular stress), p53 expression is stimulated. In turn, p53 binds to *WAF1* regulatory elements and transcriptionally activates its expression. The WAF1 protein subsequently

binds to and inhibits cyclin-dependent kinase activity, preventing phosphorylation of critical cyclin-dependent kinase substrates and blocking cell cycle progression. In tumor cells with inactive p53, this pathway would thereby be defective, permitting unregulated growth.

Experimental Procedures

Cell Culture and Transfection

The SW480-IAB3 cell line was obtained following cotransfection of SW480 cells with plasmids PG13-Gal (see below) and pCMV-Neo-Bam (Baker et al., 1990) and selection with genetecin. Individual clones were isolated by limiting dilution and tested for the presence of stably integrated intact reporter by transfection with either plasmid p53-wt or p53-143 (Kern et al., 1992) and by in situ X-Gal staining. REF-112 and MCO1 cells were obtained from M. Oren and H1299 cells were obtained from A. J. Fornace. The GM4723 (GM cells) and del4A (DEL cells) lines were passaged in Earle's minimal essential media, and log phase cells were induced with dexamethasone as previously described (Mercer et al., 1990). For transfection experiments, 1.5×10^6 cells were plated in 25 cm^2 tissue culture flasks 24 hr before transfection. A total of 5 μg of DNA and 25 μg of lipofectin (Bethesda Research Laboratories, Gaithersburg, Maryland) were used for transfections. For growth inhibition experiments (Figure 5), hygromycin (0.25 mg/ml) selection began 24 hr after transfection.

Plasmid Constructs

PG13–Luc and MG15–Luc plasmids were cloned by inserting the HindIII–EcoRI fragments containing wild-type or mutant p53-binding elements (PG13–CAT and MG15–CAT; Kern et al., 1992) into the HindIII–EcoRI sites of pBluescript II SK(+) (Stratagene, La Jolla, California). PG13 contains 13 copies of a p53-binding site, while MG15 contains 15 copies of a subtly mutated p53-binding site. The 200 bp EcoRI–BamHI fragment containing the polyoma promoter (from pBE-L.Py; Munholland et al., 1992) was cloned into pBluescript II SK(+) constructs containing either PG13 or MG15. A 2.6 kb SacI luciferase cassette or a 3 kb β-galactosidase cassette without promoter elements was then cloned downstream to create either PG13–Luc, MG15–Luc, or PG13–Gal, respectively. pC-WAF1-S (sense) and pC-WAF1-AS (antisense) expression plasmids were prepared by cloning the full-length *WAF1* cDNA as a NotI fragment from a cDNA library clone (pZL-WAF1) into the NotI site of pCEP4 (Invitrogen). The pC-WAF1-ES mutant vector was similarly obtained from a polymerase chain reaction (PCR)-generated cDNA insert, containing a G to A transition at nucleotide 222, resulting in a stop codon instead of tryptophan at amino acid 49. The 2.4 kb *WAF1* promoter region was obtained by PCR amplification using a *P1-WAF1* subclone as template and the primers 5′-CCACAAGCTTCTGACTTCGGCAG-3′ and 5′-CCCAGGAACAAGCTTGGGCAGCAG-3′. This PCR fragment was cloned into the HindIII site of pBluescript KS(+) (Stratagene) to yield plasmid pWWP. The plasmid pDM, which lacks the p53-binding element 2.4 kb upstream of *WAF1*, was obtained by digesting pWWP with SacI and recircularization. WWP–Luc and DM–Luc plasmids were cloned by inserting the 2.6 kb BamHI luciferase cassette from PG13–Luc into the XhoI sites of pWWP and pDM.

RNA Isolation and Northern Blot Analysis

Total RNA was prepared by CsCl gradient ultracentrifugation of guanidine isothiocyanate–lysed cells as described (Davis et al., 1986). Northern blot analysis was performed as previously described (El-Deiry et al., 1991) except that Quickhyb (Stratagene, La Jolla, California) was used for hybridization. The *MDM2* probe was made from a 1.6 kb cDNA fragment (Oliner et al., 1993), and the *GADD45* probe was made from a 5.0 kb genomic fragment (provided by A. J. Fornace; Kastan et al., 1992) through random priming (Feinberg and Vogelstein, 1983).

Library Screening

Poly(A)$^+$ RNA (3.5 μg) obtained from GM cells induced with dexamethasone for 6 hr was used to make an oligo(dT)-primed cDNA library with the SuperScript Choice System (GIBCO BRL Research Products Life Technologies, Grand Island, New York). A total of 100 ng of cDNA, comprising the 1.5–5 kb fraction, was ligated to λ Ziplox EcoRI arms (GIBCO BRL Life Technologies, Incorporated, Gaithersburg, Maryland), and phage clones were obtained following infection of Escherichia coli strain Y1090ZL. Phage clones were screened by hybridization of colony lifts to either subtracted or unsubtracted cDNA probes prepared as described below. Excision of pZL1 plasmid clones was carried out by phage infection of the excision strain DH10B-Zip (Elledge et al., 1991).

Unsubtracted cDNA probes were prepared from 2 μg of poly(A)$^+$ RNA "driver" using oligo(dT) as primer and Moloney murine leukemia virus SuperScript II as described (Hampson et al., 1992), except that following alkaline hydrolysis with NaOH and neutralization with HCl, the cDNA was isopropanol precipitated in the presence of 0.17 M sodium perchlorate, washed with 70% ethanol, vacuum dried, and resuspended in 10 μl of water (Kinzler and Vogelstein, 1989). Unsubtracted cDNA (20 ng) was then labeled with random primers using Sequenase as described (Hampson et al., 1992). Subtracted cDNA probes were prepared by hybridizing for 22 hr of 500 ng of target cDNA to 10 μg of poly(A)$^+$ driver RNA, chemical cross-linking with 2,5-diaziridinyl-1,4-benzoquinone (provided by J. Butler), and labeling as described (Hampson et al., 1992).

A mouse *WAF1* genomic clone was isolated by screening 1×10^6 clones of a mouse genomic DNA library in λFix II (Stratagene), using the human *WAF1* cDNA as a probe. One hybridizing clone was obtained. An 11 kb HindIII fragment containing the second exon of mouse *WAF1* was subcloned into the HindIII site of pBluescript II SK(+). An 0.3 kb PstI fragment from this clone (containing part of mouse *WAF1* exon 2) was used to probe the Northern blot in Figure 4.

Wild-Type p53-Producing Defective Adenovirus

The cDNA for p53 was obtained as a BamHI fragment from the p53-wt vector (Baker et al., 1990; Kern et al., 1992) and cloned into the BamHI site of pMV10 (Wilkinson and Akrigg, 1992). The HindIII fragment of pMV10-p53-wt was then subcloned into the HindIII site of the pMV60 vector (Wilkinson and Akrigg, 1992) to make the vector pMV60-p53-wt. The plasmids pMV60-p53-wt and pJM17 (Wilkinson and Akrigg, 1992) were cotransfected into 293 cells. Recombinants were plaque purified and tested for production of transcriptionally active p53 by infection of the SW480-IAB3 cell line. A plaque-purified recombinant (Ad-p53) induced β-galactosidase activity in infected SW480-IAB3 cells. The β-galactosidase-producing defective adenovirus (Ad-gal) was obtained from plaque-purified recombinants following cotransfection of 293 cells with pMV35 and pJM17. Both Ad-p53 and Ad-gal were further purified by CsCl banding.

Isolation of a p53-Responsive Element Using a Yeast Enhancer Trap

The *P1-WAF1* clone was digested to completion with HaeIII, AluI, or Sau3AI, subcloned into the plasmid pBM947, and used to identify p53-binding sites by genetic selection in yeast (Wilson et al., 1991; T. T. et al., unpublished data). A total of 530,000 clones were obtained in E. coli, and the DNA from these clones was used to transfect Saccharomyces cerevisiae cells containing a p53 expression vector and a *HIS3* gene under the control of p53 binding sequences (Nigro et al., 1992; Kern et al., 1992; T. T. and S. Thiagalingam, unpublished data). A total of 160,000 yeast clones were assayed for histidine prototrophy. Selection in the absence of histidine allowed the isolation of clones containing a p53 binding sequence; transcriptional activation by p53 resulted in *HIS3* production and subsequent survival of the yeast transformants. DNA was isolated from such clones and tested for induction of histidine prototrophy in yeast strains with or without human p53 expression vectors. The sequence of one of the sites is shown in Figure 6, and the sequence of the second site (greater than 8 kb upstream) was 5′-GGCCTTGCCCGGGCTTGTCT-3′.

Chromosomal Localization

A screen of human genomic *P1* clones for *WAF1* was performed using the primers 5′-CTTTCTAGGAGGGAGACAC-3′ and 5′-GTTCCGCTGCTAATCAAAG-3′ from *WAF1* exon 3 for PCR (Genome Systems, Incorporated, St. Louis, Missouri). The PCR was performed using the Bind-Aid kit (U. S. Biochemical, Cleveland, Ohio) in a 25 μl reaction containing 2.5 μl of 10× PCR buffer (U. S. Biochemical), 2 μl of 2.5 mM each dNTP (dATP, dCTP, dGTP, and TTP), 0.5 μl of Bind-Aid

(0.5 μg/μl SSB), 0.5 μl of each primer (350 ng/μl), 10 ng of DNA template, and 2 U of AmpliTaq (Perkin-Elmer Cetus, Norwalk, Connecticut). Amplification was carried out for 35 cycles (following the profile of 95°C for 30 s, 57.5°C for 1 min, and 70°C for 1 min), yielding a 99 bp PCR product. The *P1* clone obtained (*P1-WAF1*) contained approximately 85 kb, including at least 21 kb upstream of exon 1 and 7 kb downstream of exon 3 of *WAF1*. *P1-WAF1* DNA was labeled with biotin and hybridized to metaphase chromosomes as previously described (Meltzer et al., 1992). Eighteen metaphase nuclei were examined for *WAF1* localization.

Luciferase Assays

Transfected cells were washed twice with 4 ml of Dulbecco's phosphate-buffered saline per T-25 flask. The cells were lysed with 0.3 ml (per T-25) of 1 × CCLR buffer (Promega) for 10 min at room temperature. After a 5 s spin to pellet large debris, 10 μl of supernatant was added to 90 μl of reconstituted luciferase assay reagent (Promega). Light emission was detected by scintillation counting.

Isolation of WAF1 Antisera and Western Blot Analysis

A WAF1–glutathione S-transferase fusion construct was prepared in pGEX-2T (Pharmacia). Electroeluted WAF1–glutathione S-transferase fusion protein was used to immunize mice as described previously (Smith et al., 1993). Western blots using 1:500 dilution of mouse polyclonal sera were performed and analyzed as previously described (Smith et al., 1993).

Acknowledgments

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Immune Response in Drosophila

The cover shows a Drosophila fat body, stained for dorsal-related immunity factor (Dif), which is localized in the nucleus after injury. For details see the article by Ip et al. in this issue.

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Inhibition of Cellular Proliferation by Antisense Oligodeoxynucleotides to PCNA Cyclin

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The proliferating cell nuclear antigen (PCNA or cyclin) is a nuclear protein recently identified as a cofactor of DNA polymerase δ. When exponentially growing Balb/c3T3 cells are exposed to antisense oligodeoxynucleotides to PCNA, both DNA synthesis and mitosis are completely suppressed. A corresponding sense oligodeoxynucleotide has no inhibitory effects. These experiments indicate that PCNA (cyclin) is important in cellular DNA synthesis and in cell cycle progression.

THE PCNA WAS ORIGINALLY described as a nuclear antigen detectable by immunofluorescence, and its appearance has been correlated to the proliferative state of the cell (*1*). A similar nuclear protein described by Bravo and co-workers was called cyclin (*2*). PCNA and cyclin were shown to be identical (*3*) and were identified as an auxiliary protein for DNA polymerase δ (*4*), necessary for the replication of simian virus 40 DNA (*5*). A partial-length cDNA clone of human PCNA was isolated and sequenced by Almendral *et al.* (*6*), and a full-length human cDNA clone was isolated (*7*) from an Okayama-Berg library (*8*).

We have constructed oligodeoxynucleotides complementary to either the sense or the antisense strand of the first 36 nucleotides of the PCNA sequence, beginning from the AUG codon.

The relevant PCNA sequence for these experiments includes the last 48 nucleotides that precede the AUG codon, as well as the first 72 nucleotides of the coding sequence (Fig. 1). Four 18-mer oligodeoxynucleotides were synthesized (Fig. 1). The first 18-mer (cyclin 1) extends from nucleotide 4 to nucleotide 21, inclusive. Cyclin 3 extends from nucleotide 22 to nucleotide 39 inclusive. Cyclin 2 and cyclin 4 are the antisense complementary strands of cyclin 1 and cyclin 3, respectively. The oligonucleotides were made on an Applied Biosystems 380B DNA synthesizer by means of β-cyanoethyl phosphoramidite chemistry. The overall yield of 18-mer was 80 to 85%, and the lyophilized product was redissolved in culture medium.

In these experiments we used Balb/c3T3 cells, exponentially growing on cover slips in the presence of Dulbecco's medium supplemented by 10% fetal bovine serum. The oligodeoxynucleotides [a mixture of cyclins 1 and 3 (sense) or cyclins 2 and 4 (antisense)] were added at time 0 to cultures without serum, as follows. The growth medium was removed, and the cultures were incubated in Dulbecco's medium (without serum) to which oligodeoxynucleotides (30 μ*M*) were added. After incubation for 30 min with oligodeoxynucleotides, serum was added to a final concentration of 10% without changing the medium. The cells were incubated for up to 24 hours, labeled with [^{3}H]thymidine (6.7 μCi/ml) for 30 min, and then fixed in methanol. Addition of antisense oligodeoxynucleotides markedly inhibited the proliferation of Balb/c3T3 cells. Cultures incubated with cyclins 2 and 4 remained sparse, whereas the corresponding controls and the cells exposed to sense oligodeoxynucleotides grew to 70% confluence, as evaluated by visual observation.

The labeling indices of these cultures, as measured after autoradiography, are shown in Fig. 2. We show two experiments in which the labeling index of the controls (no treatment) is slightly different, but these experiments have been repeated seven times and the results are reproducible. With this protocol, the labeling index of exponentially growing control cells should remain roughly constant, with time, as was observed (Fig. 2). Cells exposed to antisense oligodeoxynucleotides had a markedly decreased labeling index. The decrease began at 6 hours, and by 16 hours no labeled cells could be detected. In cells exposed to sense oligodeoxynucleotides the labeling index remained constant, at about the same level of their controls. Cells exposed to the sense oligodeoxynucleotides had a decrease in the number of autoradiographic grains per nucleus (20 to 30; the grains in the control could not be counted), although the percentage of labeled cells remained the same as in controls. The decrease is due, we believe, to the high amounts of sense and antisense oligodeoxynucleotides added to the cultures. It is conceivable that the oligodeoxynucleotides are

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slowly broken down, thus liberating enough thymidine triphosphate (dTTP) to considerably dilute the precursor pool. The amount added, 30 μM, is above the cellular concentration of dTTP, which is in the order of picomoles (*9*).

The effect of these oligodeoxynucleotides on cell proliferation, independent of the effect on the incorporation of [^{3}H]thymidine, was also measured by counting the number of mitoses per 1000 cells (Fig. 3). Addition of sense oligodeoxynucleotides to the cultures did not change the mitotic index, which remained indistinguishable from that of the controls (no treatment). However, in cells exposed to antisense oligodeoxynucleotides there was a complete suppression of mitosis and, in fact, no mitoses were observed in cells treated for 16 or 24 hours with antisense oligodeoxynucleotides.

In other experiments we used only one oligomer, the one extending from nucleotide 4 to nucleotide 21 inclusive (cyclins 1 and 2) and the results were identical. For example, 24 hours after addition of the oligodeoxynucleotides the labeling index of control cells was 47%, while that of cells exposed to the antisense 18-mer was <1%. The mitotic index was 1.6% in control cells, but no mitoses could be observed in the antisense treated cells. The addition of a single 18-mer antisense oligodeoxynucleotide sequence (whether it was cyclin 2 or cyclin 4) gave even more inhibition than the simultaneous addition of both 18-mers. The effect on cell proliferation was even more striking, with very little growth of the sparse cells, whereas control cells (with or without sense oligodeoxynucleotides) grew vigorously. A dose-response experiment with cyclin 2 showed that 2 μM had very little effect on the growth of Balb/c3T3 cells, 5 μM gave ~50% inhibition, and 10 μM (or more) completely inhibited growth.

The amount of DNA per cell was measured by computer-operated microspectrofluorimeter in exponentially growing cells treated with cyclin 2 (*10*). Control cells or cells treated with a sense oligomer gave very close distributions: 48 to 50% of the cells had a G_1 DNA content, 40 to 42% had an S phase content, and 10% had a G_2 content. In cells treated with an antisense oligomer, G_2 cells completely disappeared, and the fraction of S phase cells tended to have lower DNA values. These data indicate that the block must occur at the G_1/S boundary or in the early S phase.

We also quantitated the expression of the PCNA protein in cells treated with antisense oligodeoxynucleotides. The amount of PCNA in control cells, and in cells exposed to either sense or antisense oligodeoxynucleotides, was determined by immunofluorescence (*11*) (Fig. 4). Cells treated with an antisense 18-mer (cyclin 2) for 24 hours show a marked decrease in the amount of nuclear PCNA. The amount of PCNA per cell was essentially the same in control cells (untreated) and in cells exposed to the sense 18-mer (Fig. 4, A and B). In cells exposed to the antisense 18-mer the amount of PCNA was about 40% that of the control cells (Fig. 4C). Bravo and Macdonald-Bravo (*12*) found that, after 24 hours of quiescence, the amount of PCNA (cyclin) was 30 to 40% that of growing cells. It seems, therefore, that the protein is relatively stable.

Reports have indicated that exposure of cells to antisense RNA to c-*fos* inhibits cell proliferation (*13*). Heikkila *et al.* (*14*) reported that human lymphocytes exposed to a 15-mer antisense oligodeoxynucleotide to c-*myc* do not enter the S phase after mitogen stimulation. Holt *et al.* (*15*) similarly found that an antisense oligodeoxynucleotide to c-*myc* inhibited cell proliferation and induced differentiation in HL-60 cells. The effect was not as dramatic as in our experiments, but they used much higher concentrations of oligomers, 30 μM versus 4 μM in their experiments. The inhibition of growth was reversible (*15*) and, as in our studies, removal of the antisense oligomer restored growth with a delay of about 36 to 48 hours (*16*).

We have shown that exposure of exponentially growing Balb/c3T3 cells to antisense oligodeoxynucleotides of PCNA completely inhibits cellular DNA synthesis and cellular proliferation. The inhibitory effect of the antisense oligodeoxynucleotides to

```
TTC CTC CTT CCC GCC TGC  CTG TAG

CGG CGT TGT TGC CAC TCC GCC  ACC

ATG TTC GAG GCG CGC CTG GTC  CAG

GGC TCC ATC CTC AAG AAG CTG  TTG

GAG GCA CTC AAG GAC CTC ATC  AAC
```

Fig. 1. Partial sequence of the PCNA gene. The coding strand includes 48 nucleotides preceding the ATG codon (the first triplet in the third row) and 72 nucleotides of the coding sequence. The synthesized 18-mer sequences are indicated by lines, either above or below the letters.

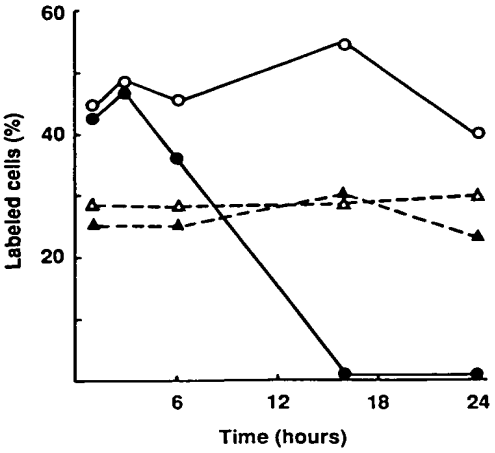

Fig. 2. Percentage of labeled Balb/c3T3 cells at various intervals after exposure to sense or antisense oligodeoxynucleotides. Cells were exponentially growing on cover slips and labeled with [^{3}H]thymidine for 30 min before fixation. ●, Cells exposed to antisense oligodeoxynucleotides; ○, their controls; ▲, cells exposed to sense oligodeoxynucleotides; △, their controls.

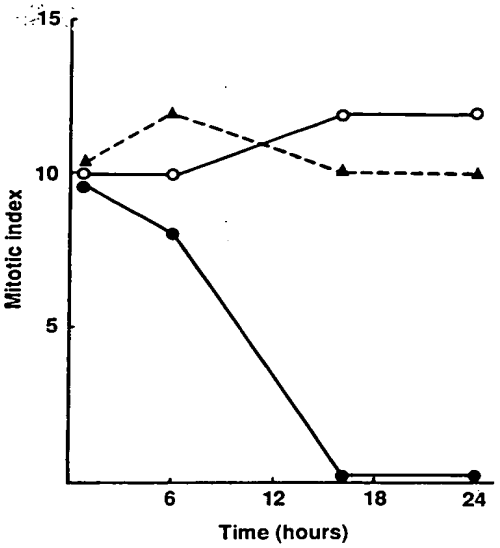

Fig. 3. Mitotic indices of Balb/c3T3 cells exposed to sense or antisense oligodeoxynucleotides. Number of mitoses per 1000 cells were determined at the indicated intervals. ●, Cells exposed to antisense oligodeoxynucleotides; ○, cells exposed to sense oligodeoxynucleotides; ▲, controls.

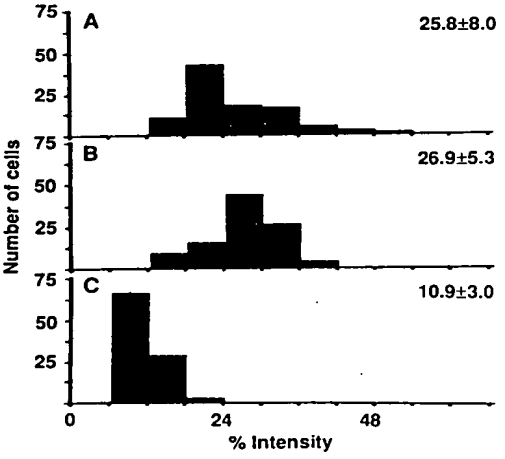

Fig. 4. Fluorescence intensity of Balb/c3T3 cells stained with a monoclonal antibody to PCNA. The abscissa is the percent fluorescence intensity as determined by a computer-operated microspectrofluorimeter. (**A**) Untreated cells; (**B**) cells treated with sense oligodeoxynucleotides; (**C**) cells treated with antisense oligodeoxynucleotides (n = 100 cells per graph).

PCNA could be due to a direct effect on PCNA or could be due to the induction of interferon. Interferons, and interferon-like substances, have been known to inhibit cell proliferation (*17*).

We cannot rule out this possibility even though it has not been shown that oligodeoxynucleotides induce interferon (*18*). PCNA is a cofactor of DNA polymerase δ, and it is believed that DNA polymerase δ is involved in cellular DNA replication (*19*). The finding that an antisense to PCNA results in complete suppression of DNA synthesis and of cellular proliferation indicates that PCNA is required for both cellular DNA synthesis and cell cycle progression. A reasonable amount of PCNA is still present in quiescent cells (*12*) or in cells inhibited by antisense oligodeoxynucleotides; the onset of cellular DNA synthesis may depend on a critical amount of PCNA. Finally, we would suggest that exposure of cells to antisense oligodeoxynucleotides may conveniently replace the more cumbersome use of antisense RNAs.

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Cachectin/TNF and IL-1 Induced by Glucose-Modified Proteins: Role in Normal Tissue Remodeling

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Proteins undergo a series of nonenzymatic reactions with glucose over time to form advanced glycosylation end products (AGEs). Macrophages have a receptor that recognizes the AGE moiety and mediates the uptake and degradation of AGE proteins. This removal process is associated with the production and secretion of cachectin (tumor necrosis factor) and interleukin-1, two cytokines with diverse and seemingly paradoxical biological activities. The localized release and action of these cytokines could account for the coordinated removal and replacement of senescent extracellular matrix components in normal tissue homeostasis.

MACROPHAGES PRODUCE THE POTENT cytokine cachectin [also called tumor necrosis factor (TNF)] in response to bacteria, viruses, and parasitic organisms (*1*). This 17-kD protein was first identified as a factor able to promote hemorrhagic necrosis in some transplanted tumors (*2*) and kill several transformed cell lines (*3*). Soon thereafter, this same protein was independently isolated (*4*) and cloned (*5*) as a mediator of cachexia and shock. A number of studies with recombinant cachectin/TNF demonstrated the pluripotent effects of this protein. Included among these diverse bioactivities are enhancement of fibroblast growth (*6*), stimulation of collagenase release from several mesenchymal cell types (*7*), bone resorption accompanied by increased osteoclast and decreased osteoblast number (*8*), promotion of angiogenesis (*9*), and induction of a number of growth factors including granulocyte-macrophage colony-stimulating factor (*10*), platelet-derived growth factor (*11*), and interleukin-1 (IL-1) (*12, 13*), itself a known growth promoter and proteolytic enzyme inducer. The paradoxical capacity of these cytokines to promote both necrotic and growth responses in tissue suggest that cachectin/TNF and IL-1 might in fact serve as the mediators of a single important biological process—normal tissue remodeling. In addition to their role during invasion, monocyte-derived macrophages are believed to play an important role in tissue homeostasis in response to senescence or local injury by regulating mesenchymal cells and turnover of extracellular matrix proteins (*14*). Although effects of cachectin/TNF and IL-1 could account for several central features of tissue homeostasis, no endogenous stimulus regulating physiologic, noncytotoxic secretion of these mediators has yet been identified.

Recently, we identified a novel membrane-associated receptor on both peritoneal macrophages and human peripheral monocytes ($K_a = 10^7 M^{-1}$, 10^5 sites per cell) that specifically recognizes proteins modified by advanced glycosylation end products (AGEs) (*15, 16*). Using different proteins such as myelin proteins and low density lipoproteins, we showed that the AGE receptor recognizes only the AGE moiety, irrespective of type of protein (*15–17*). Proteins that are enzymatically glycosylated have carbohydrate structures totally unrelated to the nonenzymatically formed AGEs and do not bind to the AGE receptor. These irreversible nonenzymatic protein modifications form, through a series of slowly occurring dehydrations and rearrangements of the nonenzymatic addition product of glucose with protein amino groups, the Amadori product (*18, 19*). One of these adducts has been identified as 2-(2-furoyl)-4(5)-(2-furanyl)-1*H*-imidazole (FFI), which forms from the condensation of two glucose molecules and two free lysine ϵ-amino groups of protein (*20*). Proteins with only Amadori-stage adducts are not recognized

Table 1. Detection of IL-1 production of human monocytes in response to AGE proteins. Normal human monocytes were prepared as described in Fig. 1. IL-1β was determined in total cell lysates by radioimmunoassay as described (*28*). Analysis of data ($n = 6$), shown here as means ± SD, by one-way analysis of variance (*32*), indicated that the difference between glucose-modified albumin (AGE-BSA) and normal unmodified albumin (NI-BSA) is statistically significant ($P < 0.002$). All other P values were also <0.001.

Ligand added	IL-1β (p*M*)
AGE-BSA (250 μg/ml)	93.5 (± 29.9)
NI-BSA (250 μg/ml)	16.2 (± 1.6)
IFN-γ (1 ng/ml)	None detected
LPS (0.2 ng/ml)	372.0 (± 36.4)

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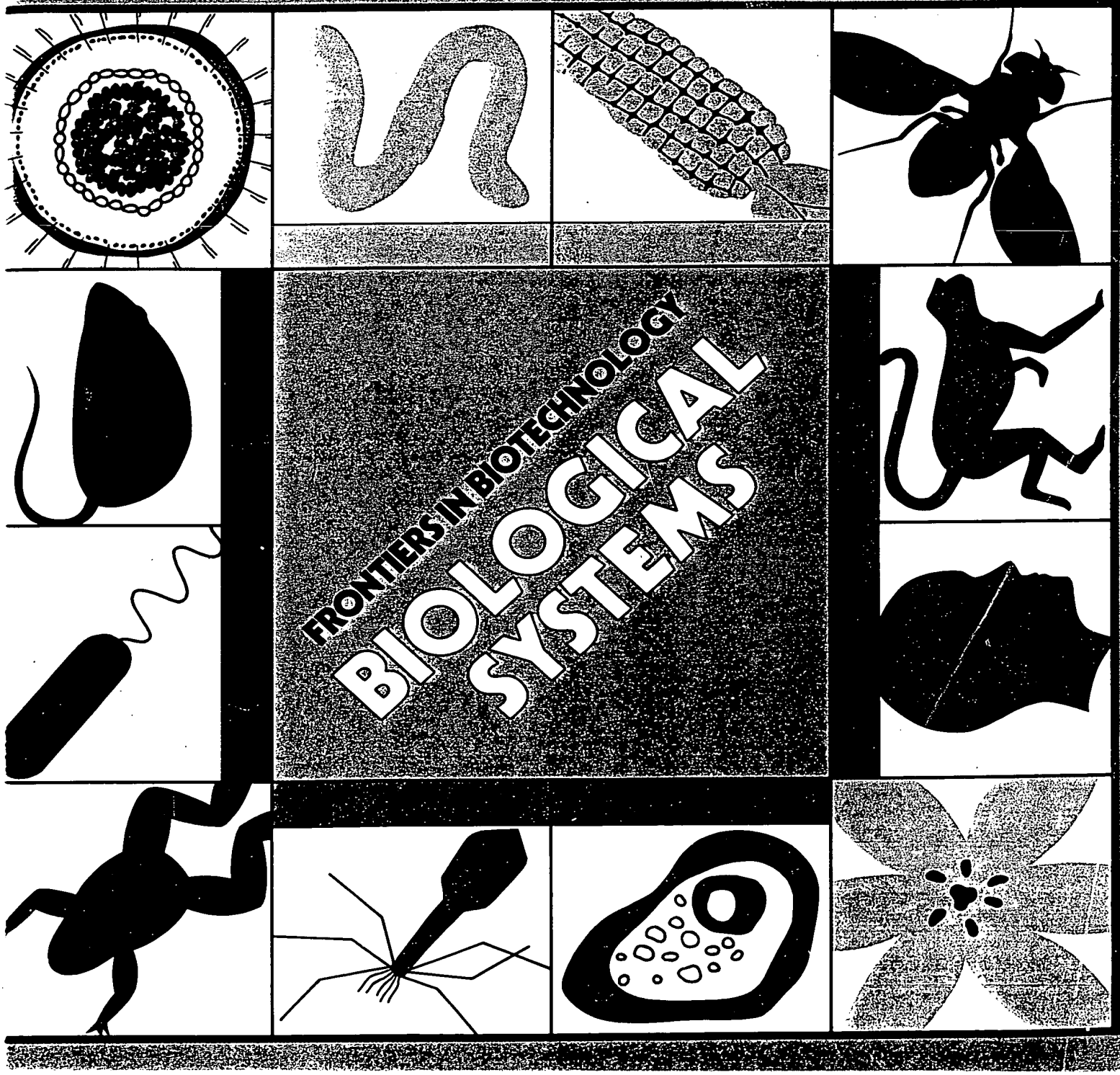

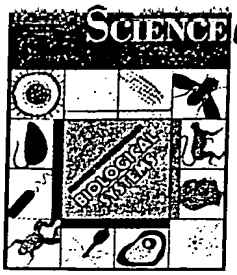

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